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Study of Tetracycline Resistance Determinants and their Genetic Supports in the Oral and Faecal Metagenomes of Six European Countries

Thesis submitted by
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ABSTRACT

Investigations of the prevalence of antibiotic resistance genes and their genetic supports are essential for our understanding of the mechanisms of resistance, and their transfer.

This study investigated the prevalence of tetracycline and erythromycin resistance in the Gram positive aerobic cultivable portion, and the total oral and faecal microbiota of six European countries. Only Gram positive isolates were investigated as they represent a distinct phylogenetic group. Furthermore, this project was part of a larger European-wide study on the biology of Gram positive organisms.

A collection of 123 tetracycline and/or erythromycin resistant isolates was made, and through macroarray analysis the most common *tet* genes were found to be *tet*(M), *tet*(O) and *tet*(W) in the aerobic oral flora, and *tet*(M), *tet*(O) and *tet*(Q) in the aerobic faecal flora. Three isolates did not hybridise to any probes on the array.

In order to investigate the contribution of the whole metagenome to antibiotic resistance, total extracted DNA was analysed on the macroarray and 12 BAC libraries were constructed. The most common *tet* genes in the oral microbiota were *tet*(M), *tet*(Q) and *tet*(30); and were *tet*(W), *tet*(O), *tet*(Q) and *tet*(32) in the faecal metagenome.

The BAC libraries were evaluated for efficiency of cloning microbial DNA, and to ensure they were representative of each microbiota, by end-sequence analysis. The libraries were screened on tetracycline. 32 resistant clones were found, only four of these were stable. One, NFtetC1, contained *tet*(O). The entire insert was sequenced to determine its support, it was shown to contain orfs with similarity to *tnpV* from Tn*4451*, and to *orf6* from Tn*916* and *cpp2* from the *tet*(O)-harbouring *Campylobacter coli* plasmid pCC31.

Clone SFtetC10 harboured *tet*(M); PCR analysis illustrated it was flanked by sequences with homology to those flanking *tet*(M) in Tn*916*, however, *int* from Tn*916* did not amplify with specific primers. Clones IStetC1 and FRStetC11 did not hybridise to any probes on the array. These harbour either novel or rare *tet* genes. Clone IStetC1 was subcloned and found to harbour a putatitve natural chimera of two tetracycline resistance plasmids: pRSB107 and pR64.

This study thus provides further evidence of the prevalence of antibiotic resistant bacteria in the human GastroIntestinal (GI) tract, and the difference in prevalence of tetrcycline resistance determinants in the aerobic cultivable flora and total microbiota.

Furthermore, it illustrates how antibiotic resistance genes are contained on mobile genetic elements which are mosaic in structure having undergone evolutionary changes in which functional modules are exchanged.

DECLARATION

I hereby certify that the work embodied in this thesis is the result of my own investigations, except where otherwise stated.

Samples of stimulated saliva and faeces from healthy volunteers were collected by Mlle Aline Launay (Faculté de Parmacie, Université Paris Sud, France), Dr. Denis Mater (INRA-UEPSD, Domain de Vilvert, France), Dr. Jaana Mättö (VTT Biotechnology, Finland), Dr. Paola Mastrantonio (Istituto di Microbiologia, Universita di Ancona, Italy), Dr. Carla Pruzzo (Istituto Superiore di Sanita, Roma, Italy), Dr. Arnfinn Sundsfjord (IMB University of Tromsó, Norway) and Dr. Andrea Patterson (Rowett Research Institute, Aberdeen, Scotland).

Macroarray experiments were carried out in conjunction with Dr. Andrea Patterson (Rowett Research Institute, Aberdeen, Scotland). Oral and faecal BAC-end sequences were produceded by Dr. Mike Quail (Wellcome Trust Sanger Institute, Cambridge, UK), other BAC insert sequencing was performed by LARK Technology Systems (Takeley, Essex, UK). Screening of the *Enterococcus faecium* 664:1H1 BAC library was carried out by Dr. Aurelie Villedieu (Eastman Dental Institute, London, UK).

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CONTENTS

List of tables

Chapter One

Chapter Two

Chapter Three

Chapter Four

Chapter Five

Chapter Six

List of Figures

Chapter One

Chapter Two

Chapter Three

ABBREVIATIONS

ABC	ATP-Binding Cassette Protein
aa-tRNA	Aminoacyl-transfer Ribonucleic Acid
ATP	Adenosine Tri-phosphate
BAC	Bacterial Artificial Chromosome
BHI	Brain Heart Infusion
BIBAC	Binary Bacterial Artificial Chromosome
BLAST	Basic Local Alignment Search Tool
Bp	Base Pair
°C	Degree Celsius
COG	Clusters of Orthologous Groups
CSP	Competence Stimulating Peptide
CTn	Conjugative transposon
DGGE	Denaturing Gradient Gel Electrophoresis
dH_2O	Distilled water
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
ESAC	*E. coli* – Streptomyces Artificial Chromosome
FISH	Fluorescent In-Situ Hybridisation
g	Gravitational Force
GI	Gastrointestinal
GTP	Guanosine Tri-phosphate
h	Hour
HGT	Horizontal Gene Transfer
HMW	High Molecular Weight
IPTG	Isopropyl-β-D-thiogalactopyranoside
IS	Insertion Sequence
Kb	Kilobase
µl	Microlitre
LB	Luria Bertani

µm	Micrometer
M	Molar
MDR	Multidrug resistance
MFS	Major Facilitator Super family
min	Minute
ml	Millilitre
MLS	Macrolide, Lincosamide and Streptogramin
mm	Millimetre
mM	Micro-molar
MMR	Mismatch Repair
MRSA	Methicillin resistant *Staphylococcus aureus*
NHS	National Health Service
nm	Nanometer
ORF	Open Reading Frame
OriT	Origin of Transfer
PBP	Penicillin Binding Proteins
PCR	Polymerase Chain Reaction
PFGE	Pulsed Field Gel Electrophoresis
RBS	Ribosome Binding Site
rDNA	Ribosomal Deoxyribonucleic Acid
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
rpm	Revolutions Per Minute
RPP	Ribosomal Protection Proteins
RT-PCR	Real Time Polymerase Chain Reaction
S (50S, 16S)	Svedberg unit
s	Second
SDS	Sodium Dodecyl Sulphate
SMR	Small Multidrug Resistance Protein Family
SSC	Standard Saline Citrate
SSCP	Single Strand Conformation Polymorphism
SSU	Small Sub Unit
TAC	Transformation-Competent Bacterial Artificial

	Chromosome
TBE	Tris Borate EDTA
TGGE	Temperature Gradient Gel Electrophoresis
Tn	Transposon
T-RFLP	Terminal Restriction Fragment Length Polymorphism
tRNA	Transfer Ribonucleic Acid
TTGE	Temporal Temperature Gradient Gel Electrophoresis
VRE	Vancomycin Resistant Enterococci
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside
YAC	Yeast Artificial Chromosome

CHAPTER ONE

Introduction

1.1 Bacteria, Disease and Treatment

1.1.1 The Discovery of Antibiotics

By the late 1800s it was generally accepted that bacteria and other microbes were the cause of various diseases. A phenomenon which at that time was known as the 'germ theory of disease' (Brock *et al.*, 2002). As a result, biologists dedicated time in the search for chemicals that would kill these bacteria without having toxic effects on the human host. In 1888, the blue pigment pyocyanase, released by *Bacillus pyocyaneus* was found to stop the growth of other bacteria in the culture, this is now recognised as the first report of an antibiotic. Unfortunately pyocyanase proved to be unstable when isolated and toxic to animals (Gaby *et al.*, 1945)

In 1929 Alexander Flemming famously discovered penicillin when bacterial agar plates contaminated with moulds showed areas of growth inhibition around the mould colonies (Fraenkel, 1998; Chain *et al.*, 2005). Flemming illustrated that the penicillium moulds produced a small compound which was able to diffuse through the agar and lyse the bacteria (Fraenkel, 1998).

Since then, numerous antibiotic compounds have been isolated from both fungi and prokaryotes, and subsequent chemical modifications of certain antibiotics have resulted in semi-synthetic derivatives (Hopwood & Chater, 1980; Monaghan & Barrett, 2006).

Antibiotics may be either bacteriostatic or bacteriocidal (Brock *et al.*, 2002). Bacteriostatic antibiotics such as the sulphonamides, tetracyclines, choloramphenicol, erythromycin and trimethoprim do not kill cells, but prevent growth by a variety of mechanisms, therefore if the antibiotic is removed growth

will resume. Bacteriocidal agents kill the organism. These include the penicillins, cephalosporins and aminoglycosides (Brock *et al.*, 2002). Antibiotics may target a single species, having a narrow spectrum of activity, or be potent against a wide variety of bacteria (broad-spectrum activity) (Powledge, 2004).

1.1.1.2 Classes of Antibiotics

1.1.1.2.1 Macrolides

1.1.1.2.1.1 Structure and Background

Macrolides are made up of 14- (erythromycin and clarithromycin), 15- (azithromycin) and 16- (josamycin, spiramycin and tylosin) membered lactones covalently attached via glycosidic bonds to amino or neutral sugars (Figure 1.1) (Bryskier *et al.*, 1993; reviewed in Roberts *et al.*, 1999b).

Erythromycin was the first macrolide antibiotic discovered, a product of *Saccharopolyspora erythraea*, and was introduced to the clinic in 1952 (reviewed in Roberts, 2002). It is used in the treatment of respiratory tract infections in adults and children, in bone and soft tissue infections and in specific cases of sexually transmitted diseases including chlamydia and syphilis (Goldman & Scaglione, 2004).

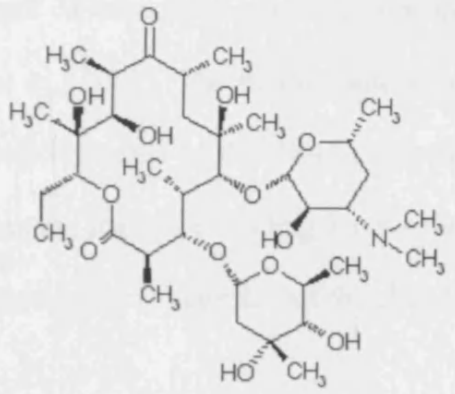


Figure 1.1: Molecular Structure of Erythromycin (Taken from Vester, 2001)

By 1953 resistance had been detected in staphylococcal isolates (reviewed in Roberts, 2002) worldwide. In general, erythromycin is much more effective against Gram positive bacteria than Gram negatives.

The other macrolides are all derivatives with synthetic substitutions on the lactone ring. These synthetic molecules confer improved stability and penetration of the drug and are less likely to interact with other antibiotics (Goldman & Scaglione, 2004).

1.1.1.2.1.2 Mode of Action

The uncharged form of macrolides is capable of crossing the membrane by passive diffusion (there is no evidence for a membrane carrier, the use of energy or the proton motive force) (Hancock & Bell, 1988; Goldman & Kadam, 1989; reviewed in Goldman & Scaglione, 2004). Entrance of the protonated form of macrolides is restricted due to strong ionic hydrogen bonding with water (Bosnar *et al.*, 2005).

Only one erythromycin molecule binds to each 50S subunit. The macrolide binding site is situated at the base of a deep cleft in the ribosome that allows the

exit of transcribed proteins from the large subunit (Weisblum, 1995; Poehlsgaard *et al.*, 2002). The binding site is in close proximity to where the aminoacyl and peptidyl ends of the tRNAs become aligned to catalyse the formation of peptide bonds, therefore binding blocks the translation reaction of the polypeptide chain elongation (Vester & Douthwaite, 2001).

1.1.1.2.2 Tetracyclines

1.1.1.2.2.1 Structure and Background

Tetracyclines were discovered in the 1940s. They are broad spectrum antibiotics and are used clinically in the treatment of atypical pneumonia, cholera, periodontal infection, acne and many other genital, local and systemic infections (Brodersen *et al.*, 2000). They have also been used in agriculture as growth promoters (reviewed in Chopra & Roberts, 2001). *Streptomyces aurofaciens*, *Sm. viridofaciens* and *Sm. rimosus* all produce tetracyclines, in addition *Sm. aurofaciens* and *Sm. rimosus* produce chlortetracycline and oxytetracycline to help out compete neighbours and as defense mechanisms (reviewed in Nelson, 1999).

Second and third generation tetracyclines: doxycycline and minocycline (Chopra & Roberts, 2001), and glycyclines (Bronson & Barrett, 2001), respectively, are semi-synthetic derivatives which are also used clinically in dermatology (acne, rosacea and perioral dermatitis) (Maibach, 1991).

Tetracyclines are constructed of linear fused tetracyclic nucleus rings to which a variety of functional groups are attached as shown in Figure 1.2.

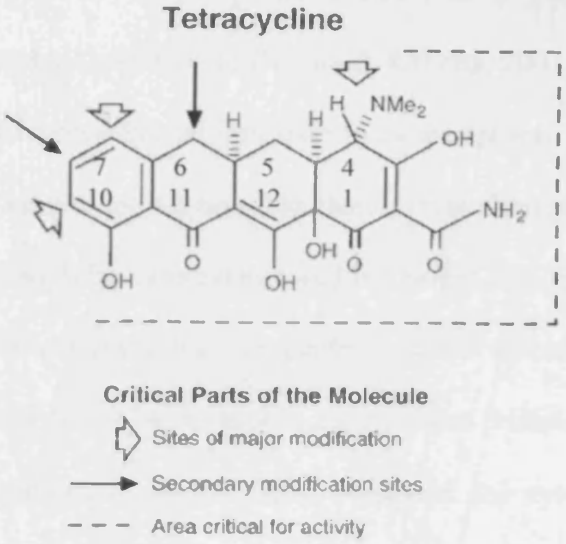


Figure 1.2: Molecular structure of tetracycline, outlining sites at which modifications of the drug occur, and the area critical for activity (www.gsbs.utmb.edu).

Each of the rings in the fused nucleus must be six membered and carboxylic (with one exception: 6-thiatetracycline which has a sulphur atom at position 6 of the C ring) (reviewed in Chopra & Roberts, 2001). The antimicrobial and pharmokinetic properties of tetracyclines are strongly influenced by their chelation of metal ions (Broderson *et al.*, 2000) which occurs at positions 1, 2, 3, 11 and 12 (amongst others) (Blackwood, 1985). The cation metal ion-tetracycline complex aids transfer across the outer membrane (through the effect of the Donnan potential (the electrical potential difference across the membrane) on the complex) and is the probable active drug species that binds to the ribosome (reviewed in Levy, 1984).

1.1.1.2.2.2 Mode of Action

Tetracycline molecules are attracted to the cell by Donnan potential across the outer membrane (Schnappinger & Hillen, 1996). They enter bacteria by passive diffusion, traversing the bacterial membrane of Gram negative bacteria through

OmpF and OmpC porin channels as a positively charged cation-tetracycline complex (reviewed in Chopra & Roberts, 2001; Lambs *et al.*, 1988). This results in an accumulation of molecule in the periplasm. The cation complex dissociates and the neutral tetracycline can then diffuse through the cytoplasmic membrane due to its lipophilic nature (reviewed in Chopra & Roberts, 2001).

Neutral tetracycline molecules transfer across Gram positive membranes in an action driven by the proton motive force (Nikaido & Thanassi, 1993).

Chelation of the antibiotic occurs in the cytoplasm due to the relatively high concentration of metal ions within the cell. This (most-likely) magnesium-tetracycline complex then binds with the 30S ribosomal subunit at one of various sites preventing protein synthesis (Lambs *et al.*, 1988). All of these sites are on or in close proximity to aminoacyl-tRNA binding site (Figure 1.3) (reviewed in Chopra & Roberts, 2001; Buck *et al.*, 1990). The binding of the tetracycline sterically interferes with the aa-tRNA binding. There are up to six sites on the 30S subunit at which a tetracycline molecule can bind, but only two are actually within the A site (the 'primary site' and the 'tet-1' site) (Versalovic *et al.*, 1997; reviewed in Brodersen *et al.*, 2000).

Within the primary site the tetracyclines do not bond with bases, but with the sugar-phosphate backbone (Brodersen *et al.*, 2000). This relative lack of specificity is thought to be the reason why tetracyclines have such a broad spectrum of activity.

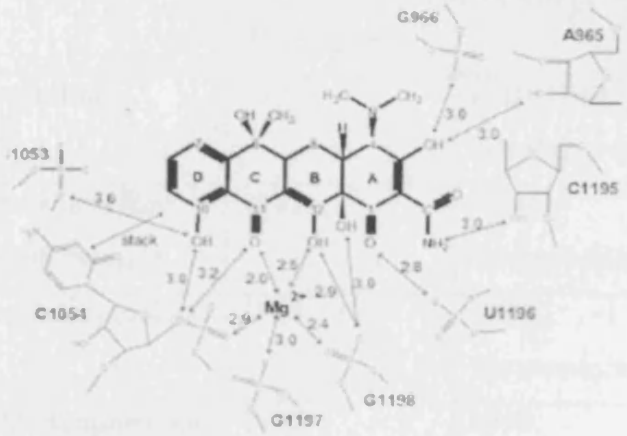


Figure 1.3: The primary tetracycline binding site (A site region) with rings A, B, C, and D of the fused-ring system and possible interactions with the 16S rRNA. The shaded area represents the positions on the tetracycline molecule that can be modified without affecting the hydonaphthacene nucleus without interefering with its inhibitory action. (taken from Hlavka *et al.*, 1985).

1.1.2 The Rise of Antibiotic Resistant Bacteria

Soon after the discovery and clinical use of antibiotics, resistance was detected (Table 1.1).

Antibiotic	Year discovered	First resistant strain	Reference / Reviewed in
Penicillin	1929	early 1940s, *Streptococcus* sp.	Schneierson, 1948.
'Protonsil Red' Sulphonamide	1932	1946, *Streptococcus* sp.	Alexander *et al.*, 1956.
Aminoglycoside	1944	1949, *Enterococcus* sp.	Scott, 1949.
Chloramphenicol	1947	1950, *Salmonella* sp.	Corda, 1950.
Chlortetracycline/ oxytetracycline	1949	1953, *Shigella* sp.	Bryson & Demerec, 1950.
Macrolides	1952	1952-53, *Staphylococcus* spp.	Pattee & Baldwin, 1962.
Vancomycin	1956	1986, *Enterococcus* spp.	Koelbl & Catlin, 1986.

Table 1.1 : The discovery of antibiotics and dates of first resistances.

Today resistance to antibiotics and other pharmaceuticals is one of the main concerns for the healthcare sector, costing the NHS millions of pounds every year (Livermore, 1994) and limiting current and future therapeutic choices. Resistance to antibiotics in pathogenic bacteria is associated with increased rates of mortality and morbidity (Austin *et al.*, 1999; Williams, 2001).

In response, repeated warnings have been given about the development of resistance and the related threats to the clinical effectiveness of antibiotics by various governing bodies (Degener, 1999; McCaig, 1995; Hoiby, 2000; Fish, 1995), and there have been many reports calling for the implementation of polices on the use of antibiotics (Stratchounski *et al.*, 2005; Bronzwaer *et al.*, 2004,

2002a,b; Casewell *et al.*, 2003; Hooton *et al.*, 2001; Cizman *et al.*, 2004) in an attempt to limit resistance.

1.1.2.1 Antibiotic Resistant Bacteria

Table 1.1 illustrates how quickly bacteria have been in responding to the selective pressures exerted in the clinical setting. These resistances have also quickly spread to the community and now resistant bacteria have been isolated from almost every environment investigated (Barbosa *et al.*, 2000; Kummerer *et al.*, 2004).

1.1.2.1.1 In Clinical Environments

Hospitals offer an environment conducive to the development and spread of antibiotic resistance. Heavy exposure to antimicrobial agents provide a strong selective pressure, and a high population density offers greater opportunities for spread compared to other environments (Tenover, 1996). Consequently, the emergence of nosocomial infections caused by multidrug-resistant bacteria is increasingly reported. Examples include MRSA strains, most of which are resistant to all antibiotics with the exception of glycopeptides (Tenover, 2004; Khan *et al.*, 2000; Moore & Lindsay, 2002). Until recently vancomycin has been used as a last line of defense against MRSA infections, however, recent decreased susceptibilities to vancomycin have been reported in Japan and the USA (Hiramatsu, 1997), and UK (Woodford, 2000). In addition, enterococci are intrinsically resistant to several commonly used antibiotics and have been shown able to acquire resistance to all currently available antibiotics either by mutations

or by transfer of mobile genetic elements (Zirakzadeh & Patel, 2006; Mascini & Bonten, 2005).

In the developed world, up to 60% of hospital-acquired infections are caused by VRE and MSRA (Morris *et al.*, 1995). They can cause a broad range of symptoms depending on the site of infection, but are commonly associated with wound infections or more seriously, blood infections (Garnier *et al.*, 2000a).

1.1.2.1.2 In The Community

In the community, the most common infections caused by antibiotic resistant bacteria are by pathogens that cause respiratory and gastrointestinal infections (Table 1.2). Some of these bacteria are likely to have spread from hospitals or farms where the high use of antibiotics has produced a strong selective pressure (Smith *et al.*, 2005). They then emerge upon sporadic use of antibiotics by out patients, and remain due to incorrect administration of the drugs (Austin *et al.*, 1999). Following their establishment they may spread amongst the population (Witte, 2004).

In addition, some multi-drug resistant pathogens have developed in the community. These include CA-MRSA (community aquired-MRSA) which exhibits different PFGE and MLST profiles to those strains which developed in hospitals, and which is emerging as a major public health concern (Witte, 2004; Drews *et al.*, 2006).

Pathogen	Disease	Antibiotic for treatment	Resistance Phenotype	Reference
Streptococcus pneumoniae	Pneumonia, Meningitis	Penicillin/ Erythromycin	PenR, ErmR, TetR, CmpR, TriR	Felmingham *et al.*, 2002 Diaz-Meija *et al.*, 2002 Gay & Stephens 2001 Montanari *et al.*, 2003 Perez-Trallero 2001a, 2001b
Streptococcus pyogenes	Respiratory tract infections	Penicillin	ErmR	Farrell *et al.*, 2006
Neisseria meningitides	Meningitis	Penicillin	ErmR, PenR	Florez *et al.*, 1997
Haemophilus influenzae	Influenza	Penicillin	PenR,	Johnson *et al.*, 2006
Moraxella catarrhalis	Respiratory tract infections	Penicillin	PenR, AmpR	Fung *et al.*, 1994 Walker *et al.*, 2000
Salmonella typhimurium	Gastroenteritis	Fluoroquinolone	FlrR	Izumiya *et al.*, 2005
Campylobacter jejuni	Gastroenteritis	Erythromycin	ErmR	W Yan *et al.*, 1991 Vaishnavi & Kaur, 2005

Table 1. 2: Common antibiotic-resistant community pathogens. Resistance phenotypes: Pen, penicillin; erm, erythromycin; tet, tetracycline; cmp, chloramphenicol; tri, trimethoprim-sulfonamide.

1.1.2.1.3 In Agriculture

The wide-spread use of antibiotics in agriculture as growth promoters has resulted in the rise of antibiotic resistant bacteria in farms (van den Bogaard *et al.*, 2000). These can then spread to the community and lead to transfer of resistance genes to pathogens (as discussed above) (Smith *et al.*, 2005). A commonly cited example is the use of avoparcin as a growth promoter in cattle farming. Avoparcin resistance

genes were soon found in bovine commensals, and studies have shown that resistance has spread to the commensal organisms of the farmers (Smith *et al.*, 2005; Casewell *et al.*, 2003; Wegener *et al.*, 1999). Such findings have led to the banning of the use of most antibiotics for this purpose in most of the developed world (Casewell *et al.*, 2003), however, the use of some tetracyclines, ionophores and glycolipids remains legal (Butaye *et al.*, 2001).

1.1.2.2 The Relationship Between Antibiotic Use and Resistance.

One of the major causes of increasing antibiotic resistance is increased selective pressure i.e. increased volume of drug use (Austin *et al.*, 1999). Evidence for the relationship between antibiotic use and resistance is widely documented and can be referred to in terms of geographical and chronological trends (Baquero *et al.*, 2002; Mueller *et al.*, 2006).

Figure 1.4 illustrates the effect of increased use of antibiotics as growth promoters in chicken broilers in Denmark before their ban, and the subsequent decrease following this ban.

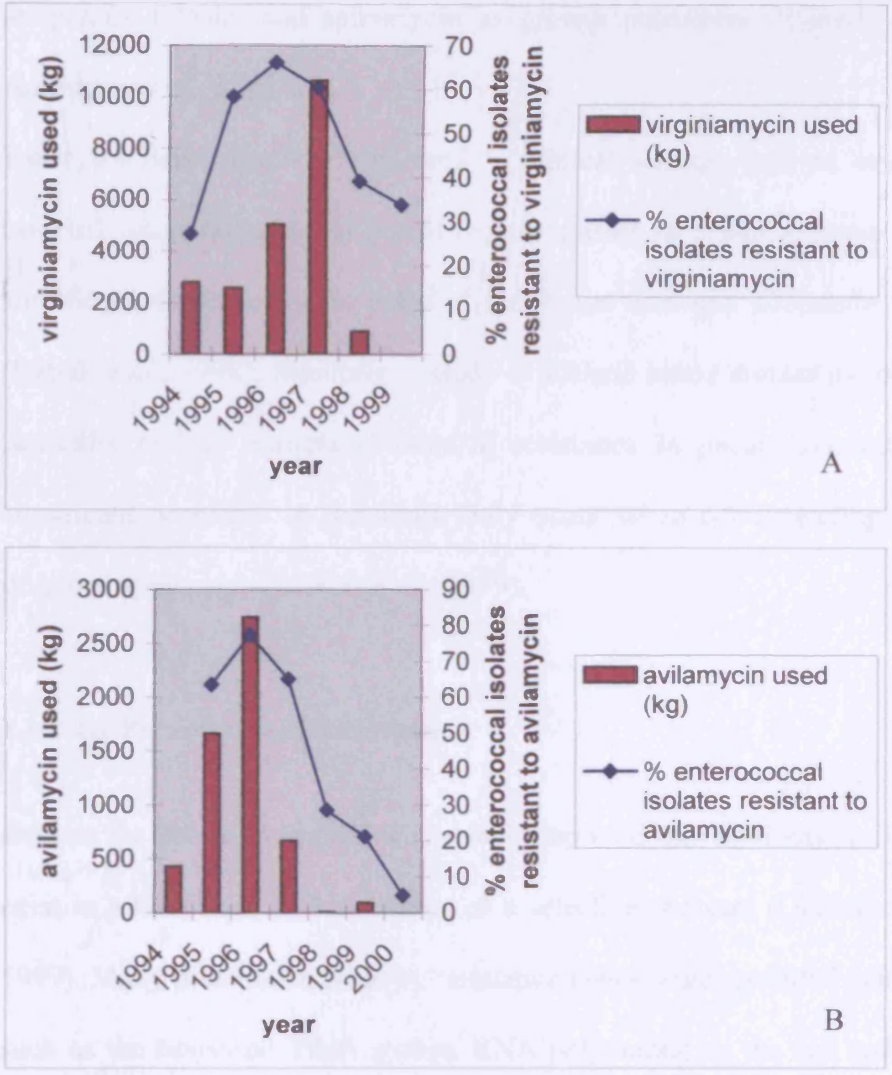


Figure 1.4 : The use of antimicrobials virginiamycin (A) and avilamycin (B) in Danish farming as growth promoters, and the levels of resistance to virginiamycin (A) and avilamycin (B) in enterococcal isolates taken from chicken broilers between the years 1994 – 2000. Data taken from Aarestrup *et al.*, 2001

In this case a decrease in resistance is observed when the selective pressure is removed. Presumably because the carriage of resistance is costly to the cell and therefore is lost in the majority of the population (Austin *et al.*, 1999). The sensitive strains can then out-compete resistant strains (Dahlberg & Chao, 2003). This pattern has also been seen in similar cases since the EU ban of the use of

avoparcin, tylosin, and spiramycin as growth promoters (Bywater *et al.*, 2005, Aarestrup *et al.*, 2001).

Examples have also been observed in clinical settings such as in a New York hospital where the phasing out of certain classes of cephalosporins resulted in a significant decrease in the rates of resistance amongst *Klebsiella* spp. isolates (Rahal *et al.*, 1998). Similarly, a study in Iceland based around the clinical use of penicillin and its associated rates of resistance in pneumonococci has shown significant decreases in resistance only occur when corresponding decreases in drug use were made (Austin *et al.*, 1999).

1.1.2.2.1 Persistence of Resistance.

Despite the above examples, it is widely reported that antibiotic resistance genes exist in a host even in the absence of a selective pressure (Guillemot & Carbon, 1999). Many proteins encoded by resistance genes target essential macromolecules such as the ribosome, DNA gyrase, RNA polymerase or the cell wall (Tenson & Mankin, 2006). Such targets usually have deleterious effects resulting in decreased bacterial fitness (increased energy costs, reduced growth rate) (Maisnier-Patin *et al.*, 2002). However, in some cases the fitness costs associated with carriage of a resistance determinant are compensated for with a mutation (N Luo *et al.*, 2005). One such example is in streptomycin resistant *Escherichia coli* which was found to undergo second site mutations that compensate for some of the fitness costs of *rpsL* mutations (which confer streptomycin resistance) over 500 generations (Bouma & Lenski, 1988; Lenski *et al.*, 1991). Such mutations convey a selective advantage over the original sensitive strains (which are then out competed), and

virtually preclude the evolved resistant lineages from reverting to drug sensitivity (Schrag *et al.*, 1997).

Selection will also favour resistant strains with enhanced survival ability or virulence (Livermore, 1993), such as those which harbour resistance determinants linked to genes that confer a favourable phenotype such as tetracycline resistant infantile colonic *E. coli* in which strains which carry *tet*(A) and *tet*(B) more often contain the genes for P fimbriae compared to tetracycline susceptible strains (Karami *et al.*, 2006; Krulwich *et al.*, 2005).

1.1.3 Types of Resistance

1.1.3.1 Intrinsic and Acquired Resistance

1.1.3.1.1 Defining Resistance

Resistance is considered if a bacterium is not susceptible to a drug, or if it possible to demonstrate a mechanism for resistance in vitro (Degener, 1999).

Resistance to antibiotics is caused by a variety of mechanisms (Livermore, 2003):

1). Presence of an enzyme that inactivates the antibiotic.

2). Presence of an alternative enzyme to that which is being acted upon by the antibiotic.

3). Mutation in the antibiotic's target site preventing or reducing binding.

4). Change in the target site caused by another protein.

5). Posttranscriptional or posttranslational modification of the antibiotic's target.

6). Reduced uptake i.e. permeability barrier.

7). Active efflux of the antibiotic.

8). Compensatory over-production of the target.

As with all genes, it is commonly considered that two resistance genes are of the same class if the exhibit ≥80% similarity in their amino acid sequence, and different if <80% (Levy *et al.*, 1999).

1.1.3.1.2 Intrinsic Resistance

Intrinsic resistance is defined as that which is present in all the members of a given species or genus (Nelson, 1999).

Many clinically important Gram negative bacteria exhibit intrinsic resistance to a number of different antibiotics (Kumar & Schweizer, 2005). In Gram negatives the outer membrane lipopolysaccharide is much less fluid than the inner membrane and charged molecules have been shown to permeate the outer membrane at up to 100 times less than the rate of the usual phospholipid bi-layers (Nikaido, 1994). Thus hydrophobic antibiotics are unable to easily cross into the cell. In addition, the porins used to translocate essential nutrients in Gram negatives use an extremely narrow aqueous diffusion channel (7-10 A in *E. coli*) and are lined with charged amino acid residues that prevent the entry of antibiotics this way (Nikaido, 1994). In *P. aeruginosa*, the passage of β-lactams is prevented due to the absence of these 'classical' porins and the presence of a low affinity porin (Parr *et al.*, 1987). In addition, in *P. aeruginosa* the up regulation of the *MexAB-OprM* operon is responsible for the activation of a multidrug resistance pump that is always expressed to some degree as part of the excretion of the quorum sensor mediator homoserine lactone (Ziha-Zarifi *et al.*, 1999). This is an example of genetically mediated intrinsic resistance (Li *et al.*, 1994) and the over-expression of such pumps can significantly raise MICs. In this case, the MIC of *P. aeruginosa* to

carbenicillin has been reported to be up to 2000 times higher than that of a mutant with no efflux pump (Nikaido, 1998).

In Gram positive bacteria, the outer peptidoglycan layer appears to offer little resistance to the diffusion of antibiotics (Schnappinger & Hillen, 1996). One exception are the Mycobacteria since, in addition to a peptidoglycan layer they also posses an outer lipid bi-layer of high order and thus lower fluidity, to prevent the diffusion of antibiotics (Philalay *et al.*, 2004; Nguyen & Tompson, 2006). However, efflux systems of Gram-positive organisms belonging to the MFS, SMR, or ABC families confer resistance to weakly lipophilic agents and organic cations (Nikaido, 1994; Putman *et al.*, 2000; van Veen & Konings, 1998).

1.1.3.1.3 Acquired Resistance

Acquired resistance is present only in certain isolates of a species or genus (Salyers & Amabile-Cuevas, 1997).

1.1.3.1.4 Origin of Antibiotic Resistance Genes

The origin of antibiotic resistance genes in pathogenic bacteria is unclear. Use of antibiotics in a clinical setting has only been widespread in the last ~60 years (Livermore, 2004b), therefore it is highly unlikely that spontaneous mutation is responsible for the creation of genes which confer resistance via mechanisms which require the cooperative action of several proteins. In contrast, efflux of antibiotics may be conferred by the mutation of existing export mechanisms (Paulsen *et al.*, 1996).

Antibiotic producing bacteria commonly encode covalent-modification enzymes to inhibit their corresponding antibiotic (Wiener *et al.*, 1998). The first identification of these self-protecting mechanisms was in *Streptomyces* sp. and soon led to the hypothesis that resistance was at least in part due to the dissemination of genes from these organisms (Davies, 1994a, 1994b). Sequence analyses have since confirmed this theory (Wiener *et al.*, 1998; Zilhao *et al.*, 1988) identifying *Streptomyces* sp. as the original hosts of: neomycin acetyltransferase (*Sm. fradiae*); viomycin phosphotransferases (*Sm. vinaceus*); vancomycin resistance determinant *vanA* (*Sm. coelicolor*) (Garnier *et al.*, 2000); ribosomal methylases conveying resistance to thiostrepton (*Sm. azureus*) and MLS antibiotics (*Sm. erythreus*) (Thompson *et al.*, 1982). In addition, beta-lactams are thought to be spread from beta-lactam-producing organisms such as filamentous fungi and actinomycetes (Liras & Martin, 2006).

1.1.3.1.5 The Role of Mutations in the Emergence of Resistance.

Mutation is one of the major contributors to antibiotic resistance in bacteria via the production of changes in existing drug targets (Hooper, 2001) or through effects on the regulatory systems (Barbosa & Levy, 2000). This results in a decrease in the affinity of the antibiotic for the targets, for example emerging linezoid resistance in enterococci is due to the mutational modification of the domain V of the 23S rRNA gene which contains the antibiotic binding site (Livermore, 1993). Furthermore, it is a key process in refining existing resistance genes, potentially increasing the MIC (Pillai *et al.*, 2001), as is the case for quinolone resistance in clinical isolates of *S. pneumoniae*: those with substitutions at Ser79 (to Phe) in

ParC and Ser81 (to Phe) in GyrA were found to have the highest MICs (Broskey *et al.*, 2000). In addition, the MICs of fluroquinolone resistant *C. jejuni* and *C. coli* were found to be increased by up to 128-fold with point mutations of *gyrA* at codon 86 (Thr-Ile) (Ge *et al.*, 2005).

1.1.3.1.6 Co-Selection of Resistant Determinants

In addition to the consequences of a direct selective pressure, the use of macrolides, including erythromycin and sulfamethoxazole-trimethoprim are also associated with selection of tetracycline resistance (Nielsen *et al.*, 2004). Such a phenomenon is termed co-selection. Co-selection occurs when antibiotic resistant determinants are linked together by their carriage on the same mobile genetic element leading to the development of multidrug-resistant strains (Nielsen *et al.*, 2004; Tenover, 2004; Khan *et al.*, 2000). The modular structure of genetic elements lends itself well to this phenomenon (Section 1.2.4) (Shapiro *et al.*, 2005). MRSA (resistant to glycopeptides, macrolides, aminoglycosides, tetracycline, rifampin, sulfonamides and pefloxacin, with reduced susceptibility to fusidic acid and fosfomycin) and VRE (resistant to aminoglycosides, vancomycin and teicoplanin) are examples of pathogenic bacteria that exhibit cross-resistance (Carias *et al.*, 1998; Moore & Lindsay, 2002; Bonten, 2001).

In addition, there is also a growing body of evidence that shows that resistance to toxic metals, such as mercury, is linked to antibiotic resistance determinants through either co-existance on the same genetic element (Davis *et al.*, 2005) or cross-resistance (resistance to metals and antibiotics being conferred by the same resistance determinant – often an efflux pump) (Baker-Austin *et al.*, 2006).

1.1.3.2 Resistance to Different Classes of Antibiotics

1.1.3.2.1 Macrolides

1.1.3.2.1.1 Classification of Macrolide Resistance

Macrolides are inhibitors of protein synthesis and are bacteriostatic (Roberts *et al.*, 1999b). Gram negative bacteria are usually intrinsically resistant to these antibiotics due to the low permeability of their membranes to the drugs (reviewed in LeClercq & Courvalin, 1991).

Three mechanisms of resistance to macrolides have been reported so far.

The methylases modify the 23S rRNA subunit at a site universal to all macrolide targets (reviewed in Roberts *et al.*, 1999b) this also confers cross-resistance to the 'MLS_B' antibiotics, including lincosamides and streptogramin B; chemically distinct, hydrophobic compounds which bind at the same site in the 50S ribosomal subunit (Vester & Douthwaite, 2001). These genes do not convey resistance to the streptogramin A-type antibiotics.

In contrast, some erythromycin resistance determinants which encode efflux pumps, and those that encode enzymatic inactivation systems (see Table 1.3) do not have the MLS phenotype of resistance (Vester & Douthwaite, 2001).

Those isolates carrying only *mef* genes are typically described as exhibiting the 'M' phenotype (Macrolide resistance) and remain susceptible to lincosamide and streptogramin B (reviewed in Roberts *et al.*, 1999b). *msr* (macrolide streptogramin resistance) genes confer resistance to the streptogramin B antibiotics in addition to the macrolides but remain susceptible to clindamycin (MS phenotype) (Johnston *et al.*, 1998).

1.1.3.2.1.2 Resistance Mechanisms

1.1.3.2.1.2.1 Methylases

Macrolides dissociate the peptidyl-tRNA molecule from the ribosome during the early stages of elongation, thus preventing protein synthesis resulting in a lack of functional ribosomes within the cell (reviewed in Vester, 2001; Roberts *et al.*, 1999b). In 1953, the first resistance to this drug was reported due to the post-transcriptional modification of the 23S rRNA by adenine–N^6-methyltransferase which adds a methyl group to a specific adenine in the 23S rRNA moiety, an important residue in the binding of MLS_B antibiotics (Vester, 2001). A number of enzymes exist in different genera and are responsible for unique modifications and variations in MIC amongst species. In the case of *E. coli* modifications to the A2058 adenine confer the highest level of resistance (reviewed in Weisblum, 1995). In *S. pneumoniae* the A2059 adenine is modified causing relatively low-level resistance, and in *H. pylori* the A2057 residue is methlyated conferring low-level resistance to 14-membered macrolides, but no resistance to 16-membered lactones (reviewed in Weisblum, 1995). Methylases have been catalogued using the *erm* designation for methylases (Erythromycin Ribosome Methylation) (Fluit *et al.*, 2001)

The *erm* alphabet was first described in detail by Marylin Roberts *et al.* in 1999. The review brought into line all previous descriptions of resistance mediated by methlytransferases which had been confusing and had incidences of the same designation being given to unrelated genes, and different names being given to the same gene.

The *erm* genes are summarised in Table 1.3.

Mode of Action	Gene	Genera in which the gene is commonly found
Methylases	*erm*(A)	*Actinobacillus, Staphylococcus, Streptococcus*
	erm(B)	*Abiotrophia, Actinobacillus, Bacteroides, Campylobacter, Clostridium, Escherichia, Enterococcus, Fusobacterium, Haemophilus, Klebsiella, Neisseria, Pediococcus, Peptostreptococcus, Prevotella, Staphylococcus, Streptococcus, Wolinella*
	erm(C)	*Actinobacillus, Bacillus, Campylobacter, Eubacterium, Haemophilus, Lactobacillus, Neisseria, Peptostreptococcus, Staphylococcus, Streptococcus, Wolinella*
	erm(D)	*Bacillus*
	erm(E)	*Streptomyces*
	erm(F)	*Actinobacillus, Actinomyces, Bacteroides, Clostridium, Eubacterium , Fusobacterium, Gardnerella, Haemophilus, Neisseria, Porphyromonas, Prevotella, Peptostreptococcus, Selenomonas, Streptococcus, Treponema, Veillonella, Wolinella*
	erm(G)	*Bacillus, Bacteroides*
	erm(H)	*Streptomyces*
	erm(I)	*Streptomyces*
	erm(N)	*Streptomyces*
	erm(O)	*Streptomyces*
	erm(Q)	*Actinobacillus, Campylobacter, Clostridium, Streptococcus, Wolinella*
	erm(R)	*Aeromicrobium*
	erm(S)	*Streptomyces*
	erm(T)	*Lactobacillus*
	erm(U)	*Streptomyces*
	erm(V)	*Streptomyces*
	erm(W)	*Micromonospora*
	erm(X)	*Corynebacterium*
	erm(Y)	*Staphylococcus*
	Clr	*Streptomyces*
ATP-Binding Transporters		
	msr(A)	*Staphylococcus*
	vga(A)	*Staphylococcus*
	vga(B)	*Staphylococcus*
Major Facilitator Superfamily Transporters		
	mef(A)	*Corynebacterium, Enterococcus, Micrococcus, Staphylococcus, Streptococcus*
	lmr(A)	*Streptomyces*
Esterases		
	ere(A)	*Enterobacter, Escherichia, Klebsiella, Citrobacter*
	ere(B)	*Escherichia, Klebsiella, Proteus*
Hydrolases		
	vgb(A)	*Enterococcus, Staphylococcus*
	vgb(B)	*Staphylococcus*
Transferases		
	vat(A)-(C)	*Staphylococcus*

	vat(D)-(E)	*Enterococcus*
Phosphorylases		
	mph(A), *mph*(B)	*Escherichia*
	*mph*I	*Staphylococcus*

Table 1.3: Erythromycin resistance determinants and their hosts (updated from Roberts, 1999; Cooper, 1996; Cousin *et al.*, 2003;).

Other methylase genes including *tlr*(D), *mdm*(A) and others (see Table 1.3) all offer resistance to 16-membered ring macrolides, however, these genes have not yet been found outside their antibiotic producing host (reviewed in Roberts *et al.*, 1999b).

As a result of these investigations, the synthesis of novel macrolides has focused on the development of drugs that target ribosomes that have already been methylated at A2058 (LeClercq *et al.*, 2002).

1.1.3.2.1.2.2 Efflux genes

A further mechanism of macrolide resistance is the efflux of the antibiotic from the cell by pumps in the cellular membrane (reviewed in Roberts *et al.*, 1999b). At present there are three classes of pumps in Gram positive cocci: the *mef, msr* and *vga* classes.

Macrolide efflux (*mef*) genes are commonly found on conjugative elements in the chromosome which are capable of intergeneric transfer (Luna *et al.*, 1999). The best characterised *mef* genes, *mefA* and *mefE* were isolated from *Sm. pyogenes* and *Sm. pneumoniae*, respectively (Clancy *et al.*, 1996; Arpin *et al.*, 1999). These genes convey the M phenotype. The encoded pumps have homology to the major facilitator superfamily (MFS) of efflux proteins (LeClercq, 2002). They exhibit

90% DNA homology and 91% amino acid homology to each other, thus are now considered to be in a single class: *mef*(A) (reviewed in Klaasen & Mouton, 2005). The mechanism of transport of the antibiotic across the membrane is via secondary active transport (transfer of molecules using the advantage of a previously existing concentration gradient, therefore no ATP is required). *mef*(A) is in the DHA3 (Drug:H^+ antiporter-3) family, which use a proton gradient as the driving force (reviewed in Roberts *et al.*, 1999b).

The *msr* (macrolide streptogramin resistance) genes are putative members of the ABC transporter superfamily and convey the MS phenotype (Reynolds *et al.*, 2003; reviewed in Roberts *et al.*, 1999b). The most common genes in this class are *msr*A and *msr*B which were both found in *Staphylococcus aureus* isolates (Reynolds *et al.*, 2003) and are both primary active transporters of the drug E1 (drug exporter 1) family. ABC transporters involved in drug efflux use ATP as an energy source (Van Bambeke, 2000). The efflux machinery contains two ATP binding cassettes. Upon hydrolysis and antibiotic binding a conformational change occurs in the hydrophobic membrane spanning domains exporting the antibiotic resulting in export of the drug (Garmory, 2004; Reynolds *et al.*, 2003).

The third class of efflux pumps consists of the *vga* (virginiamycin factor A) genes also identified in staphylococci (Allignet & El Solh, 1997). These confer resistance to the streptogramin A antibiotics (reviewed in Roberts *et al.*, 1999b).
In addition, a further efflux pump providing resistance to lincosamide only has been described in *Sm. lincolnensis*, encoded by the *lmrA* gene (reviewed in Roberts *et al.*, 1999b).

1.1.3.2.1.2.3 Other mechanisms

Other mechanisms of resistance against the individual antibiotic classes also exist including enzymes that hydrolyse streptogramin B (encoded by *vgb* and *vgb*B), and those that modify streptogramin A antibiotics by the addition of an acetyl group (encoded by *vat*, *vat*B, *vat*C, *sat*A and *sat*G) (Nakajima, 1999). Macrolides can also be affected by enzymatic inactivation. Hydrolysis of the lactone ring of the macrocyclic nucleus by EreA and EreB (Leclerq, 2002), and the addition of a phosphate on the 2'-hydroxyl-group of the amino sugar by the type I (MphA) and type II phosphotransferases have been reported in *Enterobacteriacaea* and *S. aureus* (reviewed in Roberts *et al.*, 1999b; Ounissi & Courvalin, 1985; Arthur *et al.*, 1986).

1.1.3.2.1.2.4 Regulation of Resistance

Inducible expression of the *erm* genes can occur in the presence of erythromycin, and in some cases lincosamides or streptogramin B (Weisblum, 1995). This is dependent on altered secondary structure upstream of the gene in the presence of the antibiotic in a mechanism involving translational attenuation (Gryczan *et al.*, 1980; Werckenthin *et al.*, 1999; reviewed in Weisblum 1995).

1.1.3.2.2 Tetracyclines

1.1.3.2.2.1 Classification of Resistance

Resistance to tetracyclines has developed over time with significant increases only apparent since their wide use in clinical and environmental settings (reviewed in Chopra & Roberts, 2001). Tetracycline resistance can be mediated through a

variety of mechanisms. Ribosomal protection and efflux of the drug across the cell membrane are common. However, enzymatic inactivation has also been reported, and some genes have yet to be assigned a mechanism (reviewed in Roberts, 2005). Genes encoding resistance are all designated *tet*, for tetracycline resistance unlike the macrolide resistance genes which have different designations according to resistance mechanism (Levy *et al.*, 1999).

The increasing number of *tet* genes has meant that the method of nomenclature using the letters of the alphabet is insufficient and numbers from 30 onwards are now used for novel genes. *otr* (oxytetracycline resistance) genes also exist. These are used to designate those genes first identified in oxytetracycline producing organisms, however, there is no inherent difference between tetracycline and oxytetracycline resistance genes (reviewed in Chopra and Roberts, 2001; Speer *et al.*, 1992).

To date 23 efflux genes (20 *tet* genes, 2 *otr* genes and 1 *tcr* gene (tetracycline resistance gene from a tetracycline-producing *Streptomyces* sp.); 11 Ribosomal Protection Protein (RPP) genes (10 *tet* genes, 1 *otr* gene); three enzymatic (all *tet* genes) and one gene of unknown mechanism (*tet*(U)) have been reported (Table 1.4).

Class of tetracycline resistance gene	Gene	Genera where the gene is commonly found
Ribosomal protective proteins	*tet*(M)	*Eikenella, Kingella, Neisseria, Bacteroides, Fusobacterium, Haemophilus, Veillonella, Pasteurella, Abiotrophia, Arcanobacterium, Bacillus, Butyrivibrio, Gemella, Mycoplasma, Ureaplasma, Actinomyces, Aerococcus, Bifidobacterium, Gardnerella, Corynebacterium, Eubacterium, Bacillus, Listeria, Staphylococcus, Clostridium, Peptostreptococcus, Eneterococcus, Streptococcus, Acinetobacter, Afipia, Enterobacter, Erysipelothrix, Escherichia, Klebsiella, Lactobacillus, Lactococcus, Microbacterium, Mitsuokella, Mycobacterium, Neisseria, Prevotella, Porphyromonas, Ralstonia,*

		Photobacterium, Pseudomonas, Selenomonas, Streptomyces, Vibrio
	tet(O)	*Campylobacter, Butyrivivrio, Aerococcus, Lactobacillus, Mobilunocus, Staphylococcus, Peptostreptococcus, Enterococcus, Streptococcus, Megasphaera, Neisseria*
	tetB(P)	*Clostridium*
	tet(Q)	*Capnocytophaga, Prevotella, Mitsuokella, Selenomonas, Porphyromonas, Bacteroides, Veillonella, Garnerella, Lactobacillus, Mobiluncus, Eubacterium, Clostridium, Peptostreptococcus, Neisseria*
	tet(S)	*Listeria, Enterococcus, Streptococcus, Streptococcus, Veillonella*
	tet(T)	*Streptococcus*
	tet(W)	*Butyrivibrio, Mitsukella, Selenomonas, Porphyromonas, Fusobacterium, Bifidobacterium, Actinomyces, Aranobacterium, Bacillus, Clostridium, Lactobacillus, Mitsuokella, Megasphaera, Neisseria, Prevotella, Porphyromonas, Roseburia, Selenomonas, Streptococcus, Streptomyces, Veillonella*
	tet(32)	*Clostridium*
	tet(36)	*Bacteroides, Clostridium, Lactobacillus*
Inactivating enzymes	*tet*(X)	*Bacteroides*
	tet(37)	Unknown
Efflux pumps	*tet*(A)	*Edwardsiella, Plesiomonas, Proteus, Pseudomonas, Serratia, Citrobacter, Klebsiella, Shigella, Salmonella, Aeromonas, Vibrio, Escherichia, Acinetobacter, Haemophilus, Veillonella*
	tet(B)	*Erwinia, Moraxella, Pantoea, Treponema, Providencia, Plesiomonas, Enterobacter, Marheimia, Proteus, Serratia, Citrobacter, Klebsiella, Shigella, Salmonella, Aeromonas, Vibrio, Escherichia, Haemophilus*
	tet(C)	*Enterobacter, Proteus, Pseudomonas, Serratia, Citrobacter, Klebsiella, Shigella, Salmonella, Vibrio, Escherichia, Aeromonas, Chlamydia*
	tet(D)	*Yersinia, Edwardsiella, Pleisomonas, Enterobacter, Citrobacter, Klebsiella, Shigella, Salmonella, Aeromonas, Vibrio, Eschericia, Pasteurella, Alteromonas*
	tet(E)	*Aeromonas, Alcaligenes, Escherichia, Providencia, Pseudomonas, Vibrio*
	tet(F)	unknown
	tet(G)	*Escherichia, Marheimia, Pasteurella, Providencia, Pseudomonas, Salmonella, Vibrio*
	tet(H)	*Acinetobacter, Actinobacillus, Marheimia, Moraxella, Pasteurella*
	tet(I)	*Escherichia, Providencia*
	tet(J)	*Proteus*
	tet(K)	*Eubacterium, Haemophilus, Norcardia, Bacillus, Listeria, Staphylococcus, Clostridium, Peptostreptococcus, Enterococcus, Streptococcus, Mycobacterium, Streptomyces, Lactobacillus, Norcardia, Streptomyces*
	tet(L)	*Fusobacterium, Veillonella, Actinomyces, Bacillus, Listeria, Staphylococcus, Clostridium, Peptostreptococcus, Enterococcus, Streptococcus, Mycobacterium, Streptomyces, Actinobacillus, Morganella, Norcardia, Salmonella, Veillonella*
	tetA(P)	*Clostridium*
	tet(V)	*Mycobacterium*
	tet(Y)	*Escherichia*
	tet(Z)	*Corynebacterium*
	tet(30)	*Agrobacterium*
	tet(33)	*Corynebacterium*
Mg^{2+} dependent resistance	*Tet*(34)	*Aeromonas, Pseudomonas, Serratia, Vibrio*
Unknown mechanism	*tet*(U)	*Enterococcus*

Table 1.4 : Tetracycline resistance determinants and their host ranges (adapted from Roberts, 2005; Guardabassi *et al.*, 2000).

1.1.3.2.2.2 Resistance Mechanisms

1.1.3.2.2.2.1 Efflux Genes

The efflux genes are well characterised. The protein products belong to the major facilitator superfamily (MFS) and are membrane associated proteins of approximately 6 kDa (Guay *et al.*, 1993). The N-terminal halves of MFS proteins show a high degree of homology to each other and have been implicated in proton translocation, whereas the C-terminal halves differ due to their predicted role in substrate recognition (Paulsen *et al.*, 1996). For transport to occur, the tetracycline molecule needs to be part of a metal-tetracycline complex. Mg^{2+} ions have been implicated as the divalent cation required (Yamaguchi *et al.*, 1991).

Part of the antibiotic binding site is in the transmembrane helix 4, which fluctuates between high and low fidelity states (Hirai *et al.*, 2003; Allard & Bertrand, 1993). The binding of the drug induces a conformational change in the gating site which opens and closes with the affinity change of the binding site (Hirai *et al.*, 2003). The H^+ transfer site is where the external proton will bind, the affinity of which is also probably affected by the opening and closing of the gate (Yamaguchi *et al.*, 1991).

1.1.3.2.2.2.2 Ribosomal Protection Protein Genes

The Ribosomal Protection Proteins (RPPs) are all approximately 72.5 kDa soluble cytoplasmic proteins (reviewed in Roberts, 2003; Taylor & Chau, 1996). The most common *tet* genes encoding RPPs are *tet*(M), *tet*(O) and *tet*(W) which encode proteins of approximately 650 amino acids (reviewed in Roberts, 1996; Burdet, 1991). The amino acid sequences of these proteins are all closely related to

translational elongation factors (Aminov *et al.*, 2001; Connell *et al.*, 2003b; reviewed in Taylor & Chau, 1996). In particular, their N-terminal regions are similar to the GTPases of EF-Tu and EF-G (Sanchez-Pescador *et al.*, 1988; Kjeldgaard & Nyborg, 1992). EF-G is a translocase which catalyses the translocation step of protein synthesis involving a ribosomal conformational change in order to move the mRNA molecule (Spahn *et al.*, 2001). EF-Tu is involved in the delivery of the aa-tRNA to the A site through the hydrolysis of GTP (Spahn *et al.*, 2001). It is believed that the RPPs have evolved from the elongation factors to function in the presence of tetracycline (Connell *et al.*, 2003).

Tet(M) and Tet(O) dislodge the tetracycline molecule from the A site to free it for the aa-tRNA to enter (Dantley *et al.*, 1998) (Figure 1.5). Tet(O) primarily binds with the ribosome at helix 34 of the 16S subunit (Spahn *et al.*, 2001) producing a conformational disturbance and causing the dissociation of the tetracycline molecule resulting in a return to the ribosomal elongation cycle (Connell *et al.*, 2002). Once Tet(O) has itself dissociated from the ribosome the entry of the EF-Tu-GTP-aa-tRNA complex into the A site is permitted. However, the Tet(O) induced conformation persists after it has left the ribosome to prevent the rebinding of tetracycline molecules (Connell *et al.*, 2003a).

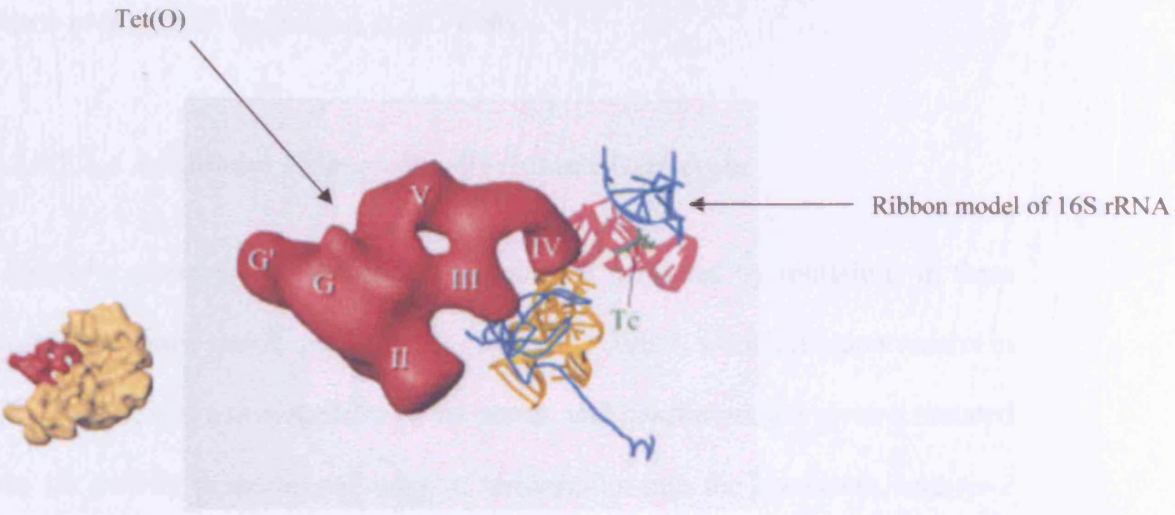


Figure 1.5 : Comparison of the binding site of Tet(O) (A) and tetracycline (B). Diagram shows a ribbon model of the 16SrRNA molecule (colour coded: Yellow, helix 18; cyan, helix 31; pink, helix 34; blue, protein S12) and Tet(O) or tetracycline (green). Tet(O) binds in close proximity to the tetracycline binding site, interacting with helix 34 and helix 18 of the 16S rRNA. Tetracycline binding is primarily at helices 34 and 31 therefore it is likely that Tet(O) displaces tetracycline molecules by a local disturbance of helix 34.
Small diagram shows the position of tet(O) with regard to the whole 16S rRNA molecule (taken from

1.1.3.2.2.2.3 Enzymatic Inactivation and Genes of Unknown Function

tet(X) encodes an NADP-requiring oxidoreductase of 43.7 kDa that inactivates tetracycline (Speer *et al.*, 1991). It was originally isolated from *Bacteroides fragalis* from Tn*4351* and Tn*4400*, however, since the enzyme requires oxygen to function, it has only been found to convey resistance in aerobically grown *E. coli* (Speer *et al.*, 1991).

tet(U) conveys a low-level MIC to both tetracycline and minocycline (reviewed in Roberts, 2003). It encodes a 11.8 kDa protein which has some similarity (21% amino acid similarity) to *tet*(M) at the carboxy terminus, however, this does not include the GTP binding domain (Ridenhour *et al.* , 1996). Because of this similarity it is thought to be RPP-like. Little else is known about *tet*(U) which was

discovered in an *Entercoccus faecium* isolate on the pK010 plasmid (reviewed in Roberts *et al.*, 1991; Ridenhour *et al.*, 1996).

1.1.3.2.2.2.4 Additional Tetracycline Resistance Genotypes

In *Neisseria gonorrhoeae* resistance can also be mediated by mutations in three endogenous genes (*mtrR*, *penB*, *tet*-2) (Hu *et al.*, 2005). *mtrR* mutation results in over expression of a non-specific efflux pump, and *penB* mutation gives a mutated porin 1B leading to decreased entry of tetracycline into the bacterium, and *tet-2* mutation results in a mutant ribosomal protein S10 causing a reduction in the binding of the drug (Hu *et al.*, 2005).

In addition, mutations in the *rrn* genes of *H. pylori* offer a degree of tetracycline resistance associated with reduced binding of the drug to the primary site (Nonaka *et al.* 2005; Dailidiene *et al.*, 2002; De Stasio *et al.*, 1989; Heuer *et al.*, 1987).

1.1.3.2.2.2.5 Regulation of Resistance

In the absence of tetracycline a repressor protein is present as a homodimer that binds to the tandemly orientated *tet* operators, blocking transcription (Su *et al.*, 1992; Orth *et al.*, 2000). In the presence of a tetracycline-Mg^{2+} complex expression ensues due to a conformational change in the repressor homodimer brought about through its binding with the tetracycline complex (Figure 1.6) allowing the binding of RNA polymerase to the operator (Blake *et al.*, 2003).

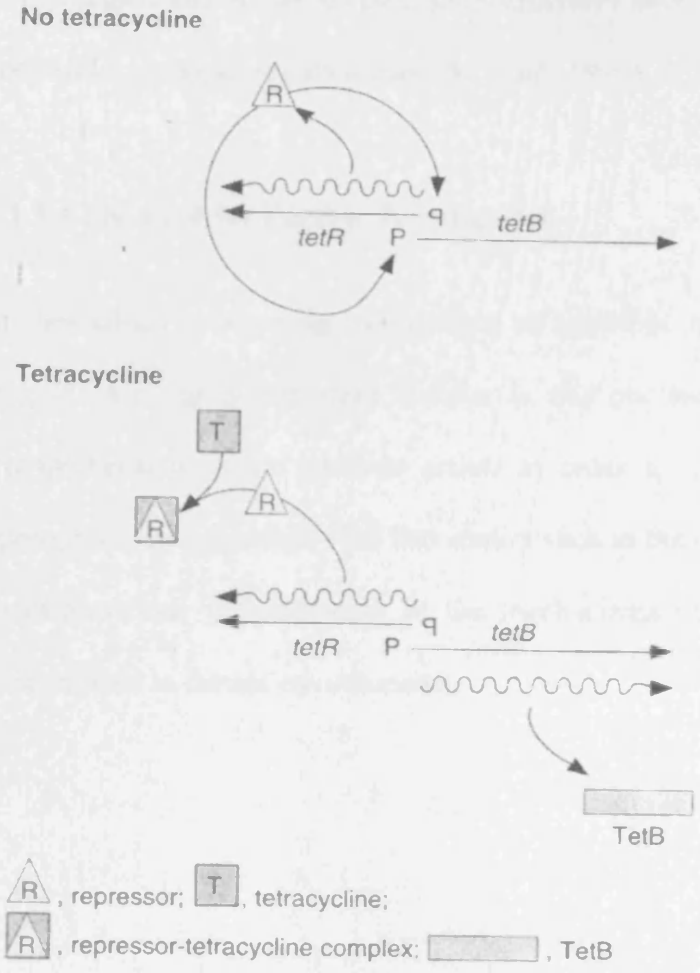


Figure 1.6 : Repressor mediated regulation of the efflux type of tetracycline resisatnce genes (www.nsm1.utdallas.edu)

tetK and tetL do not have associated cognate repressor proteins. *tet*(L) plasmid-based resistance is regulated in a similar way to that of the *erm* genes i.e. through translational attenuation (Stasinopolous, 1998).

The RPP genes are also believed to be regulated by the presence of tetracycline (Burdett, 1991), with the upstream regions of the genes implicated due to their homology within classes (Wang & Taylor, 1991; reviewed in Roberts, 1996). Northern blot analyses have detailed transcripts of varying lengths (short and long)

of this region and numerous stem-loop structures have been identified indicating a potential transcriptional attenuator (Su *et al.*, 1992).

1.1.3.4 The need for Further Investigation

Studies aimed at reporting mechanisms of antibiotic resistance and their genetic supports are hugely important in order to stay one step ahead of the pathogens. Comprehension should precede action in order to ensure the action taken is appropriate. It is therefore vital that studies such as the current work be undertaken to improve our understanding of the mechanisms of antibiotic resistance that predominate in certain environments.

1.2 Dissemination of Antibiotic Resistance Genes

1.2.1 Antibiotic Resistance and the Discovery of Bacterial Gene Exchange

At the beginning of the 1950s when the potential of antibiotics was only just being realised it was believed that resistance would not become a problem. It was thought mutation rates were not high enough, and no consideration was given regarding the ability of bacteria to exchange genetic information (Rice, 2000).

Tatum and Lederberg published evidence for genetic exchange in 1947. Their studies on mixed cultures of *E. coli* mutants had unexpectedly revealed that, having ruled out spontaneous mutantion and transformation of DNA from the culture medium, some form of bacterial 'sexual exchange' had occurred to complement the mutants under investigation (Tatum & Lederberg, 1947).

Since then, the evidence for horizontal gene transfer has been both observational and circumstantial (Kurland *et al.*, 2003; reviewed in Burrus *et al.*, 2002; Chen *et al.*, 2005). Many experiments have demonstrated the transfer of resistance phenotypes and specific mobile elements between different bacteria or the transformation of bacteria with exogenous DNA (Wang *et al.*, 2002; Guiney & Hasegawa, 1992). Additional evidence for horizontal gene transfer comes from analysis of the DNA sequences of microbial genomes (Hall *et al.*, 2005). Differing G+C content and codon usage patterns within a genome can point to DNA of foreign origin (reviewed in Lawrence & Ochman, 1997). Horizontal gene exchange can account for a significant amount of a bacterium's genome (Philippe & Douady, 2003; reviewed in Joan Curcio & Derbyshire, 2003). The percentage of genes within a genome that appear to have been horizontally transferred varies from ~1.5 – 14.5 %; for example, horizontally transferred genes account for 1.56

% of the *Borrelia burgdorferi* genome (Garcia-Vallve *et al.* 2000), 11 % of the genome of *Clostridium difficile* strain 630 (Sebaithia *et al.*, 2006), and 14.47 % of the *B. subtilis* genome (Garcia-Vallve *et al.*, 2000) thus demonstrating the role that genetic exchange has had in bacterial evolution.

It is now known that the increase in antibiotic resistance is mostly due to the transfer of determinants throughout a population, across both species and genera, followed by clonal expansion (Dutta *et al.*, 2002; Gill *et al.*, 2005). In addition, the concern about the 'predicted phenomenon' of multiple resistance is no longer unfounded due to the ability to concentrate multiple resistance determinants within a circumscribed mobile genetic region (reviewed in Kurland *et al.*, 2003; Gill *et al.*, 2005; Sekiguchi *et al.*, 2005).

1.2.2 Mechanisms of Horizontal Gene Transfer

There are three mechanisms of horizontal gene transfer (HGT), all of which are responsible for the spread of antibiotic resistance. They are transformation, conjugation and transduction.

1.2.2.1 Transformation

Bacterial transformation is the process by which cells take up 'naked', extracellular DNA from their surrounding environment, and the heritable incorporation of the DNA into the host genome (reviewed in Dubnau, 1999). This may include chromosomal DNA which is incorporated into the chromosome by homologous recombination (Ochman *et al.*, 2000), or plasmid DNA which, if it

contains an origin of replication that is recognised by the host DNA polymerase, will replicate independently (Daley *et al.*, 1998; Thomas & Neilsen 2005).

A bacterium's ability to take up DNA is termed 'competence'. In the environment, many factors are linked with the development of competence including nutrient availability, pH, temperature and calcium concentration (Bott and Wilson, 1968). It occurs naturally in some bacteria (including *Streptococcus mutans*, *S. pneumonia*, *N. gonorrheae*, *Actinobacillus spp.*, *Legionella pneumophila*, and *B. subtilis*) at a specific stage of their life cycle (Stone & Kwaik, 1999; Soloman & Grossman, 1996).

The process enables the bacterium to acquire novel genes encouraging variation and contributing to evolution (reviewed in Dubnau, 1999). Conversely, recombination of the DNA taken up may disrupt functional genes or activate defective prophage leading to the death of the cell (reviewed in Chen & Dubnau, 2004). In addition, the supplementary metabolic load conferred by plasmids may cause selection against the transformants in the environment (reviewed in Chen & Dubnau, 2004).

Transformation is also a means of acquisition of nutrients and nucleotides for use in replication for certain bacteria (*B. subtilis*, *Streptococcus* spp., *Acinetobacter calcoaceticus*, *Haemophilus* spp. and *Neisseria* spp.) (reviewed in Lorenz & Wackernagel, 1994), and rates of DNA uptake have been observed to increase in conditions of nutrient limitation (Lorenz & Wackernagel, 1991; Page & Sadoff, 1976)

The uptake of DNA into a bacterial cell begins with its binding and proceeds with its transport into the cytoplasm. Two main mechanisms for these processes have

been described, one for Gram positive organisms, the other for Gram negative organisms.

1.2.2.1.1 In Gram positive Organisms

In Gram positive bacteria double stranded DNA forms a non-covalent association with a specific DNA binding site on the cell surface (*S. pneumoniae* have approximately 30-80 of these binding sites per cell). Single stranded and glycosylated DNAs do not bind. Immediately after binding the cell surface receptor employs endonuclease activity to introduce single strand breaks in the DNA approximately 6 kb apart (reviwed in Dubnau, 1999). Transfer into the cell is energy dependent and is initiated by subsequent double strand breaks. Once inside the cell the single stranded DNA complexes with a 19.5 kDa protein to protect it from nucleases (reviewed in Dubnau, 1991). The non-transported strand is degraded.

1.2.2.1.2 In Gram negatives

In various Gram negative systems the binding of both double stranded and single stranded DNAs occurs, but is limited to DNA homologous to host DNA in the systems studied (reviewed in Dubnau, 1999). This homology facilitates the binding to a receptor protein via a recognition sequence. Once bound the DNA becomes DNase sensitive and is taken up in a 'transformasome', a membrane vesicle which apparently forms where the inner and outer membranes fuse, before it is released as a single stranded molecule into the cytoplasm (in some species, the remaining strand is degraded as in Gram positive bacteria) (reviewed in Dubnau,

1999; Dreiseikelmann, 1994). Within the transformasome the DNA is protected from restriction and nuceolytic enzymes.

The integration of DNA into the chromosome is similar in both systems. It integrates into homologous regions, catalysed by a RecA-like protein, to form a heteroduplex by the displacement of the complimentary resident strand (reviewed in Dubnau, 1999). Before replication any mismatches are repaired. This mechanism of repairing only small stretches of DNA mismatches suggests that intra-genera transfer is preferential at homologous, conserved loci (Majewski & Cohan, 1999).

1.2.2.2 Transduction

Transduction is the movement of DNA from one bacterium to another using bacteriophage as a vector (Ubukata *et al.*, 1975; Witte, 2004; Weinbauer, 2004; Weinbauer & Rassoulzadegan, 2004). This may be 'generalised' which is the incorporation of non-bacteriophage DNA, which may be chromosomal or plasmid, into the phage head with subsequent transfer to another bacterium, or 'specialised' which describes the incorporation of chromosomal DNA adjacent to the integrated phage in a transfer event (Canchaya *et al.*, 2003; Witte, 2004).

Transduction is a significant process in HGT. It has been estimated that phage mediate the transfer of between 10^{25} – 10^{28} Kb of DNA per year in the world's oceans (Rohwer & Edwards, 2002).

The mechanism of transduction requires cells to be sensitive to the infecting phage (Canchaya *et al.*, 2004), however, unlike conjugation cell-to-cell contact is not

required since the phage particle can persist in the environment as they are resistant to many physical and chemical agents (reviewed in Lorenz, 1994; Witte, 2004).

1.2.2.3 Conjugation

Conjugation is the movement of DNA from one bacterium to another in a process that involves stable cell-to-cell contact (Figure 1.7) and metabolically active donor and recipient strains for the associated replication of DNA molecules (Ankenbauer, 1997; Christie & Vogel, 2000; reviewed in Grohmann *et al.*, 2003). In Gram negative bacteria physical contact is by the sex pilus, an extracellular filament (Figure 1.7) (Lanka & Wilkins, 1995; Christie & Vogel, 2000). The means of establishing cell-to-cell contact in Gram positive bacteria has yet to be established however there is no evidence for the involvement of pili (reviewed in Grohmann *et al.*, 2003). Among the mechanisms of HGT conjugation offers the broadest host range (reviewed in Lederberg *et al.*, 1986). It not only mediates DNA transfer between bacterial genera, but also between bacteria and eukaryotes including between *Agrobacterium* and various plants (Ti plasmid) (Bevan & Chilton, 1982) and between bacteria and *Saccharomyces sp.* (for example, transfer of pAY205 and pAY201 from *E. coli* to *S. cerevisiae* (Nishikawa *et al.*, 1992); and transfer of pEK2 from *E. coli* to *Kluyveromyces lactis* (Hayman & Bolen, 1993)) (Heinemann & Sprague, 1989).

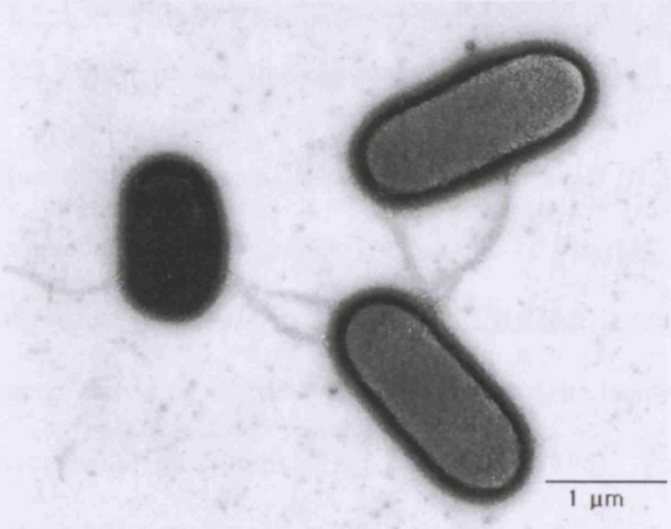


Figure 1.7: Electron micrograph of Gram negative sex pilus formation during conjugation (www.agen.ufl.edu).

In Gram negative bacteria the pilus retracts to bring the cells into close contact, or mediates contact through non-specific hydrophobic reactions (Figure 1.7) (Christie, 2001). In the majority of Gram positive bacteria the means of achieving cell contact has yet to be determined, although in some species mating pairs are formed by the production of aggregating substances (Maas *et al.*, 1998).

Conjugation results in the movement of a number of different mobile genetic elements (reviewed in Vogel & Christie, 2000; Grohmann *et al.*, 2003; Christie, 2001; Mullany *et al.*, 2002). Conjugative plasmids and conjugative transposons encode all the proteins required for their own transfer (Mullany *et al.*, 2002). In addition, they can mobilise co-resident plasmids, and transposons either in *trans* (by providing the mating apparatus) (Flannagan & Clewell, 1991), or in *cis* (by formation of a co-integrate)(Ankenbauer, 1997).

1.2.2.3.1 Mobile Elements

1.2.2.3.1.1 Insertion Sequences

Insertion Sequences (IS) elements are small (~2.5 kb), phenotypically cryptic sequences of DNA which are capable of inserting into multiple sites of a target molecule (reviewed in Mahillon & Chandler, 1998). They generally consist of a gene encoding a transposase and are delineated by terminal inverted repeats (reviewed in Mahillon & Chandler, 1998). The two terminal repeats are recombinationally-active DNA, and are involved in the transposition of some elements (Derbyshire *et al.*, 1987; Johnson & Reznikoff, 1983). The Tpase binds to the terminal repeats and the C terminal-end processes integration of the element in the host chromosome (Machida & Machida, 1989; reviewed in Mahillon & Chandler, 1998). The integration of two copies of the same element either side of a chromosomal gene can result in the formation of a composite element (an element composed of two or more other elements), for example *tet*(M) is contained on Tn*5385* which is a composite element of Tn*5381* flanked by copies of IS*1216* (reviewed by Rice *et al.*, 2002).

1.2.2.3.1.2 Integrons

Integrons (Figure 1.8) are non-conjugative elements composed of a 65 bp integration site for the capture of gene cassettes, a promoter and an integrase gene, *intI*, which catalyses the orientation-specific integration of gene cassettes by site-specific recombination (reviewed in Ploy *et al.*, 2000; Bennett, 1999; Collis & Hall, 1992; Nield *et al.*, 2001). The gene cassettes may include an antibiotic resistance genes resulting in the formation of a multidrug resistance structure driven by the integron promoter (Collis & Hall, 1995; Rowe-Magnus *et al.*, 2002;

Partridge *et al.*, 2000). Integrons are sometimes contained within transposable elements, allowing them to move to conjugative elements thus permitting their spread (Di Conza *et al.*, 2005; Raadstroem *et al.*, 1991).

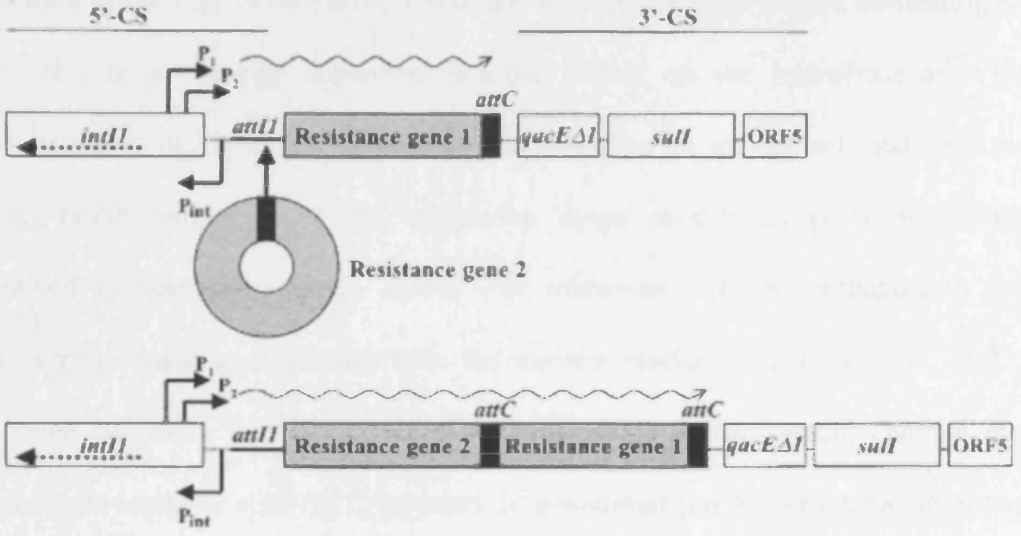


Figure 1.8: Diagram of a class 1 integron and associated gene cassette acquisition. Circularised gene cassettes insert at the attI site. P_1 and P_2 are integron associated promoters, P_{int} is the integrase gene promoter. 5'-CS and 3'-CS refer to the strand direction of the host and indicate the orientation in which the integron inserts. The *qacEΔ1* and *sulI* genes confer resistance to quaternary ammonium compounds and sulfonamides, respectively (taken from Fluit & Schmitz, 1999).

1.2.2.3.1.3 Plasmids

Plasmids are usually double stranded circular DNAs, that contain their own origin of replication, oriT, thus replicate independently from the host genome and may exist as multiple copies within one host (reviewed in Grohmann *et al.*, 2003). Conjugative plasmids harbour all the genes necessary for their transfer to a recipient cell (Willetts & Wilkins, 1984; reviewed in Grohmann *et al.*, 2003).

For the IncP transfer system of the broad host range plasmid pRP4, initiation of transfer begins with a protein complex known as the relaxosome (a mutliprotein complex constisting of both plasmid-encoded and chromosome-encoded proteins (Furste, 1987)) binding to the plasmid *oriT* (Climo *et al.*, 1996). The DNA relaxases of the relaxosome have a helicase activity and catalyse the unwinding of the DNA in an energy dependent manner reliant on the hydrolysis of ATP (Grohmann *et al.*, 2003). It then nicks the strand to be transferred, and initiates rolling-circle replication of the remaining donor strand (Llosa *et al.*, 2002; reviewed in Grohmann *et al.*, 2003). The relaxosome remains attached to the transferring strand and interacts with the transfer machinery (Llosa *et al.*, 2002; reviewed in Lanka & Wilkins, 1995). Upon arrival at the mating channel the relaxosome interacts with the C-terminus of a coupling protein which mediates the transfer of the DNA through the mating channel in an energy-dependent manner (Llosa *et al.*, 2002). The TraI proteins remain bound to the 5' plasmid terminus and are transferred with the DNA to protect the DNA from exonucleolytic activity (Llosa *et al.*, 2002; reviewed in Grohmann *et al.*, 2003). Once in the recipient, the single stranded DNA is replicated, resulting in a complete plasmid (reviewed in Grohmann *et al.*, 2003; Christie, 2001).

Many conjugative/mobilisable plasmids have been found to harbour antibiotic resistance genes (Walters, 1999), some of which are detailed in Table 1.5 and are implicated in the spread of resistance amongst pathogenic and commensal bacteria.

Plasmid	Hosts	Resistance Genes	Reference
pK214	*Lactococcus lactis*	CmpR, SmR,TetR, MLS	Perreten *et al.*, 1997
pIP501	*E. faecalis*	ErmR, CmpR, KanR, TypR, RoxR, NeoR, SmR, ClmR, LinR, AzR, ClaR.	Teuber *et al.*, 2003.
pAMβ1	*S. agalactiae, E. faecalis, S. lactis, C. acetobutylicum*	CmpR, MLS	Yu *et al.*, 1986
pRE25	*E. faecalis*	CmpR, MLS	Teuber *et al.*, 2003
pSK41	*S.aureus*	BlmR, GmR, KanR, NeoR, TobR	Firth *et al.*, 2000
pGO1	*S. aureus*	BlmR, GmR, KanR, NeoR, TobR, TmpR	Thomas Jr. *et al.*, 1989
pCF10	*E. faecalis*	TetR	Mori *et al.*, 1988
pAM180	*C. acetobutylicum*	TetR, ErmR	Bertram *et al.*, 1989
pMG1	*S. aureus, S. epidermidis*	MupR	Bastos *et al.*, 1999
PMLE300	*L. fermentum*	ErmR	Gfeller *et al.*, 2003

Table 1.5: Conjugative plasmids conferring antibiotic resistance from unicellular Gram-positive bacteria.
Abbreviations: Az, azithromycin; Blm, bleomycin; Cla, clarithromycin;Clm, clindamycin; Cmp, chloramphenicol; Erm, erythromycin; Gm, gentamycin; Kan, kanamycin; Lin, lincomycin; MLS, macrolide, lincosamide, streptogramin B antibiotics; Neo, neomycin; Sm, streptomycin; Tet, tetracycline; Tob, tobramycin, Tmp, trimethomprim; Typ, typosin. R, resistant

1.2.2.3.1.4 Transposons

Transposons are segments of DNA that are capable of excising and inserting into a new position within the host chromosome or that of a recipient cell (Twiss *et al.*, 2005; Joan Curcio & Derbyshire, 2003; reviewed in Rice, 1998a 1998b; Burrus *et al.*, 2002). They harbour a range of different genes, including antibiotic resistant genes (tables 1.6 and 1.7), however, they lack the genes for transfer and replication (reviewed in Burrus *et al.*, 2002).

1.2.2.3.1.5 Conjugative Transposons

Conjugative transposons are sequences of DNA that encode proteins for their own transfer. They are extremely common and have been found in virtually all bacteria in which they have been looked for (reviewed in Mullany *et al.*, 2002; Salyers, 1995; Clewell & Flannagan, 1995). They are not able to replicate autonomously, but do so when inserted into co-resident plasmids or the host chromosome (reviewed in Burrus *et al.*, 2002; Scott *et al.*, 1995). Their mechanism of transfer involves the excision of a double stranded covalently closed circular intermediate from the donor genome, the transfer of a single strand of the DNA through the mating channel, synthesis of a complementary strand in both donor and recipient, and the insertion of the transposon into the recipient genome either randomly or at specific sites depending on the specificity of the CTn (reviewed in Burrus *et al.*, 2002; Lu, 1994). For example, Tn*916* integrates into a large number of sites in *E. faecalis* (although preferably A+T rich), but in *C. difficile* strain CD37 it integrates preferentially into only one site, *att916* (H Wang *et al.*, 2002).

The nomenclature for transposons and conjugative transposons is confusing. Some conjugative transposons have a Tn designation which does not account for their ability to transfer, for example Tn*916* (Senghas *et al.*, 1988). The addition of a 'C' prefix has been suggested to denote those which are conjugative but this system is not universally followed as there is no longer a central database issuing Tn numbers (Campbell *et al.*, 1979). Furthermore, the numbering of transposons appears to be random with consecutive numbers being assigned whether or not the transposon is conjugative: for example, Tn*1545* is conjugative, but Tn*1546* is not

(Clewell *et al.*, 1995; Arthur *et al.*, 1993). To overcome this problem, a new database was set up at the start of 2006 (http://www.ucl.ac.uk/eastman/tn/).

1.2.2.3.1.5.1 Tn*916*

Tn*916* (Figure 1.9) has been reported to transfer between a broad range of both Gram positive and Gram negative organisms (Bertram *et al.*, 1991; Clewell *et al.*, 1995; Rice, 1998). Excision is mediated by the transposon-encoded proteins Int and Xis. The circularisation of Tn*916* is required for the expression of the transfer genes via a system that is regulated by the presence of tetracycline (Celli & Trieu-Cout, 1998). The circular form of the transposon is nicked at *oriT* prior to transfer and a single strand is transferred (Hinerfeld & Churchward, 2001). Once in the recipient the single strand acts as a template for synthesis of the complementary strand (Salyers *et al.*, 1995; Abbani *et al.*, 2005). This repaired covalently closed molecule contains *attTn*, the attachment site at which intergration occurs. Integration is mediated by Int (Storrs *et al.*, 1991). The presence of Tn*916* in the recipient cell's genome does not significantly reduce its ability to acquire further CTns of the same family (Norgren, 1991).

1.2.2.3.1.6 The Evolution of Mobile Elements

The evolution of mobile elements is through the exchange of functional modules (Frost *et al.*, 2005; Osborn & Boltner, 2002; reviewed in Bennett, 2004). This is certainly the case for Tn*916* and its relatives. For example the Tn*5397* and Tn*916* conjugation, tetracycline resistance and regulation modules exhibit high homology. However, their recombination modules are unrelated (Figure 1.9) (Roberts *et al.*, 2001a).

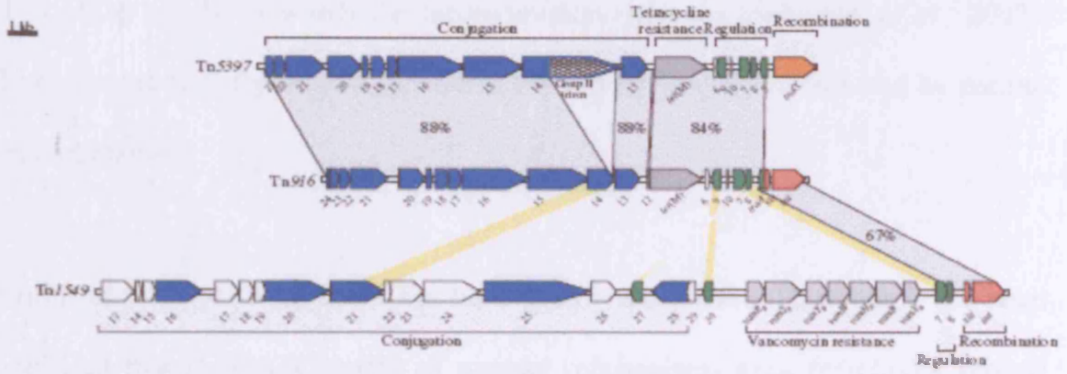


Figure 1.9: Diagrammatic representation of CTns Tn*916* and Tn*5397*, and Tn*1549*. Arrowed boxes represent ORFs (the direction of the arrow indicates the direction of transcription). The percentage similarity is shown between different functional modules. (Burrus *et al*, 2002).

Such exchanges can also occur between different types of mobile elements (Osborn & Boltner, 2002). For example, the site-specific element ICEStl from *S. thermophilus* encodes a restriction modification system related to that encoded by the *L. lactis* plasmid pKR233, and a putative conjugation module related to that of Tn*916 (*Burrus *et al.*, 2000, 2001).

The insertion of one element into another, followed by recombination events and deletions can lead to the formation of novel genes and genetic elements (Rice & Carias *et al.*, 1998).

The *erm*(33) gene demonstrates the extent of genetic rearrangements in elements. Its sequence suggests it is a product of a recombination event between an *erm*(C) and an *erm*(A) gene (Schwartz *et al.*, 2002). The first 284 bp of *erm*(33) show 99.3

% identity at the nucleotide level to *erm*(C) from plasmid pSES21, whereas the last 403 bp show 100 % identity to *erm*(A) from Tn*554*. Between these sequences in a 45 bp region at which the recombination occurred (Schwartz *et al.*, 2002). This was the first report of a functional erythromycin gene constructed by natural recombination.

Unfortunately, relatively few CTns have been characterized. However, it has been predicted that if such a variety of transfer mechanisms exist for closely related transposons it is probable that a great many more exist (Wicker *et al.*, 2003; Stokes *et al.*, 2001). Furthermore, the exchange of functional modules suggests more are evolving all of the time (Pasquali *et al.*, 2005; reviewed in Rice, 2002).

1.2.3 Transfer of Antibiotic Resistance Genes

1.2.3.1 Transfer of Macrolide- and Tetracycline Resistance Determinants

Many antibiotic resistance genes are contained on mobile genetic elements (MGEs) (Cooper *et al.*, 1996). Erythromycin and tetracycline resistance determinants have been found on a range of mobile elements (tables 1.6 and 1.7) and their transfer in the GI tract is discussed in section 1.3. However, it is important to realise that upon integration into a new host a gene may not express well or indeed at all (Wenzel & Muller, 2005; Gustafsson *et al.*, 2004). For example, the so-called 'Gram negative tetracycline resistance genes' which have only been reported in Gram negative hosts do not express well in Gram positives (reviewed in Roberts, 2002).

Erm gene	Mobile Element	Type of Element	Homology to / functional modules shared with	Hosts	Reference
erm(F)	CTnDOT	CTn	CTnERL, Tn*4351*, Tn*4551*, Tn*4399*	*Bacteroides*	Whittle *et al.*, 2001
	pBF4	Plasmid		*Bacteroides*	Shoemaker, 1985
	pBI136	Plasmid		*Bacteroides*	Smith & Macrina, 1984
	PBFTM10	Plasmid. Composite element flanked by IS	IS4351	*Bacteroides*	Tally *et al.*, 1982
erm(B)	CTnBST	CTn	*Clostridium perfringens*	*Bacteroides*	Gupta *et al.*, 2003
	Tn*5398*	Mobilisable genetic element	Tn*916*	*Clostridium difiicile* (various strains), *S. aureus, B. subtilis*	Adams *et al.*, 2002. Farrow *et al.*, 2001
	Tn*917*	Tn	pAD2	*E. faecalis*	Okitsu *et al.*, 2005
	pRE25	Plasmid	pIP501	*E. faecalis*	Teuber *et al.*, 2003
	pIP501	Plasmid	pRE25	*S. agalactiae*	Teuber *et al.*, 2003
	Tn*3692*	Transposon		*Lactobacillus crispatus*	Stroman *et al.*, 2003
erm(B), *tet*(M)	Tn*3872*	CTn	Tn*916*	*Abiotrophia defectiva*	Poyart *et al.*, 2000
erm(B), *tet*(M), *aphA-3*	Tn*1545*	CTn	pAM77	Broad host range	Okitsu *et al.*, 2005 Seral *et al.*, 2000
erm(C)		CTn		*S. aureus*	Spiliopoulou *et al.*, 2004
	pSES22	Plasmid		*S. saprophyticus*	Hauschild *et al.*, 2005

Table 1.6: Examples of Mobile Genetic Elements harbouring the most Common Erythromycin Resistant Genes.

tet Gene	Mobile Element	Type of Element	Homology to	Hosts	Reference
tet(M)	Tn*916*-Tn*1545* family	CTn		*E. faecalis, S. pneumoniae.* Broad host range	Flannagan *et al.*, 1994. Lancaster *et al.*, 2004. Poyart *et al.*, 2000, 1995b. Agerso *et al.*, 2002.
	Tn*5397*	CTn	Tn*916*-Tn*1545* family	*C. difficile E. faecium*	H Wang *et al.*, 2000 Agerso *et al.*, 2006
	CW459*tet*(M)	CTn? Tn?		*C. perfingens*	A Roberts *et al.*, 2001
tet(B)	Tn*10*	Transposon		*E. coli*	Chalmers *et al.*, 2000
	pPAT2	Plasmid	Tn*10*	*P. aeruginosa*	Hillen, 1994, Kehrenberg & Schwarz, 2001
	pHS-tet	Plasmid	pCCK3259	*Haemophilus*	Guerra *et al.*, 2002; Lancashire *et al.*, 2005
Multi-drug	PUO-stVR2	Plasmid		*S. enterica*	Guerra *et al.*, 2002
tet(W)	TnB*1230*	CTn		*B. fibrisolvens*	Melville *et al.*, 2004
tet(A)	Tn*1721*	Transposon	Class 1 integrons	Broad host range	Agerso & Sandvang, 2005 Ribera *et al.*, 2003

Table 1.7: Examples of Mobile Genetic Elements Harbouring Tetracycline Resistance Genes.

In conclusion, bacteria containing antibiotic resistance genes are ubiquitous. They have been reported in clinical, veterinary and environmental isolates, in Gram positives and Gram negatives, in commensals (as a reservoir of resistance) and pathogenic bacteria.

1.2.4 The need for Further Investigation

As predicted by Wicker *et al.*, (2003) many mobile elements exist that have yet to be discovered. In addition, the transfer of certain antibiotic resistance genes has been observed, but the elements which supports them have yet to be identified.

It is through the characterisation and understanding of mobile elements and their transfer mechanisms that we are able to predict host ranges and establish routes of transmission. This is especially important if the element harbours an antibiotic resistance gene(s). Studies have shown the importance of commensal organisms as a reservoir of resistance genes and the potential for them to spread to pathogenic bacteria. Furthermore, following characterisation of specific elements, genetic linkages of different resistances can be identified, and an appropriate clinical response to pathogens possessing such elements can be established.

1.3 The Microbiota of the Human GastroIntestinal Tract

1.3.1 The Oral Microbiota

The normal microbiota of the oral cavity consists of a wide range of organisms (Table 1.8). A recent estimate of its diversity by William Wade put the number of different species present at ~800 (William Wade Personal communication; Wade *et al.*, 2002). Approximately 50% of the oral microbiota have not been grown on artificial culture media (Munson *et al.*, 2004).

The oral cavity provides a welcoming environment for bacterial colonisation due to its ambient temperature (36°C), suitable pH (7.0-7.4), and the presence of water, nutrients and growth factors (Wilkins *et al.*, 2003). The microbiota is in a dynamic state (Morhart & Fitzgerald, 1976). Repeated periodic flushing of bacteria to the stomach (where they are usually destroyed by the acidic pH) by swallowing; brushing and flossing; and through the contact it has with other individuals' oral microbiota through kissing etc. constantly alters the composition of the microbiota (Marsh, 1999; Paster *et al.*, 2001). In addition, food-borne bacteria often pass through the oral cavity increasing diversity and providing an input of novel genetic material (Eaton & Gasson, 2001). This dynamic state of the microbiota has a knock-on impact on the conditions, and thus a dynamic equilibrium is established. Certain species are exclusive to different sites (microenvironments) within the mouth, for example approximately one third of bacterial species found on the tongue dorsum have not been found at other oral sites (Kazor *et al.*, 2003).

The host specific and site-specific nature of diversity does not lend itself well to the development of diagnostic tests for oral diseases (Bowden, 1997), this coupled

with the unknown portion of the microbiota makes the treatment of such disease difficult (Prescott Harley Klein, 2005).

1.3.1.1 Colonisation of, and Bacterial Diversity in, the Oral Cavity

As with the rest of the GI tract, colonisation of the oral cavity occurs within hours of birth with bacteria from the mother (vaginal, oral, and skin microbiota) (Fehervary *et al.*, 2004) and food-bourne bacteria (Martin *et al.*, 2003) and further develops throughout childhood. Initially aerobic species such as *Streptococcus* spp., *Neisseria* spp., *Actinomyces* spp., and *Lactobacillus* spp. predominate (Prescott Harley Klein, 2005). The anaerobic portion of the microbiota colonises on the eruption of the teeth due to the opportunity for them to occupy the space between the teeth and gums (Marsh, 2003a, 1999).

In healthy adults, by far the most predominant species are the streptococci. At least 18 different species have been isolated, most of which are from the mitis group such as *S. salivarius*, *S. mutans*, *S. pneumoniae*, *S. oralis*, *S. mitis* and the anginosus group; with viridans group streptococci predominating in dental plaque (Tanner, 2000; Bryskier, 2002).

Most bacteria in the oral cavity are from the Proteobacteria, the Gram positives, the spirochaetes or the flavobacter-bacteroides group. Isolates from the other six bacterial phyla have not been found in the oral cavity (Table 1.8).

Genus	Species
Eikenella	*E. corrodens*
Kingella	*K. dentrificans*
Neiserria	*N. sica*
Actinobacillus	*A. actinomycetemcomitans*
Haemophilus	*H. aphrophilus, H. paraphrophilus, H. segnis, H. influenzae, H. parainfluenzae, H. parahaemolyticus*
Campylobacter	*C. rectus, C. concisus, C. curvus, C. sputorum, C. showae, Bacteroides gracilis*
Selenomas	*S. sputigena, S. flueggei, S. noxia, S. infelix, S. dianae, S. artemidis*
Centipeda	*C. periodontii*
Mitsuokella	*M. dentalis*
Veillonella	*V. Parvula, V. atypical, V.dispar*
Clostridium	*C. malenominatum, C. ramosum, C. sporogenes*
Eubacterium	*E. branchy, E. nodatum, E. saburreum, E. timidum, E. saphenum, E. yurii*
Peptostreptococcus	*P. micros, P. anaerobius*
Bacillus	*B. cereus*
Lactobacillus	*L. oris, L. pantarum, L. salivarius, L. acidophilus, L.grasseri, L.casei, L. fermentum*
Atopobium	*Lactobacillus rimae, L. minutes*
Staphylococcus	*S. epidermidis, S. aureus, S. capitis, S. haemolyticus, S. hominis,, S. saprophyticus, S. simulans*
Streptococcus	*S. oralis* group/viridans group (including *S. mitis), S. intermedius* group, *S. mutans* group, *S.salivarius* group
Gemella	*G. haemolysins, G. morbillorum*
Mycoplasma	*M. salivarium, M. orale, M. buccale*
Rothia	*R. denticariosa*
Bifidobacterium	*B. dentium*
Propionibacterium	*P. acnes, P.avidum, P. granulosum, P. propionicus*
Actinomyces	*A. georgiae, A. meyeri, A. odontolyticus, A. israelii, A. naeslundii*
Micrococcus	16S matches to no known species
Stomatococcus	*S. mucilaginosus*
Corynebacterium	*C. matruchotii*
Treponema	*T. denticola, T. vincentii, T. pectinovorum, T. socranskii*
Porphyromonas	*P. gingivalis, P. endodontalis, P. asaccharolytica*
Prevotella	*P. oralis, P. veroralis, P. buccalis, P. oulora, P. buccae, P. oris, P. intermedia*
Bacteroides	*B. heparinolyticus, B. zoogleoformans*
Capnocytophaga	*C. ochracea, C. sputigena, C. gingivalis*

Table 1.8: Most commonly reported bacterial species from the human oral cavity. Species most associated with diseased states are in blue. (Tanner *et al.*, 2000; Paster *et al.*, 2001; Marsh, 1999, 2003a; Prescott Harley Klein, 2005)

1.3.1.2 Plaque

The formation of plaque illustrates how the organisms of the oral cavity exist in various microenvironments and niches that best suit their needs. Plaque is a biofilm, an organised microbial system consisting of bacterial cells associated with a surface and fixed in an extracellular matrix (Figure 1.10) (reviewed in Addy *et al.*, 1992; Guggenheim *et al.*, 2001), in which viable bacteria are mixed with dead cells (Auschil, 2001; Munson *et al.*, 2004). The biofilms that form on teeth are usually several cells thick compared to those formed on gingival tissues which are mostly monolayers due to the constant shedding of cells (Kolenbrander, 2000; Munson *et al.*, 2004).

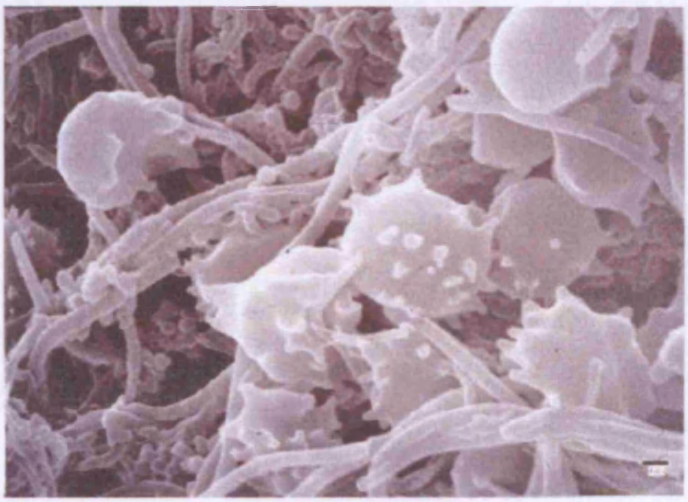


Figure 1.10: High definition Electron Scanning Micrograph of Human Dental Plaque illustrating the co-aggregation of bacterial cells and extracellular matrix. Taken from www.medicdirect.co.uk

It is estimated that ~415 species are associated with subgingival plaque (Aas *et al.*, 2005; Addy *et al.*, 1992; Paster *et al.*, 2001), of these ~40% are thought to be novel phylotypes (van Wilkelhoff & Boutaga, 2005; Paster *et al.*, 2001). Known pathogens such as *P. gingivalis, Tan. forsythensis* and *T. denticola* are reported in

the majority of samples, but typically as only a minor component of plaque. In one molecular study using 16S rDNA libraries to detect diversity in the oral cavity, 215 unknown phylotypes were identified; 33 were found to be cultivable strains that had not yet been characterised and 182 were uncultivated (Perea *et al.*, 2004).

The advantages for the bacterial cells of biofilm formation include protection from the environment (Mah *et al.*, 2001), nutrient availability through metabolic cooperation, and the acquisition of new genetic traits (in *S. mutans* transformation within an active biofilm occurs at a rate of 10- to 600-fold higher than with planktonic cells) (Davey, 2000; Molin & Tolker-Nielsen, 2003; Roberts *et al.*, 2001b; Luo *et al.*, 2005).

The protection offered by biofilms includes shelter from antibiotics and antiseptics, with bacteria less susceptible by a factor of up to 1000 compared to their planktonic counterparts (Zheng & Stewart, 2002; Marsh, 2003b). This protection is thought to be afforded by the exopolymer matrix of plaque which has the ability to exchange ions with antibiotics and thus prevent their diffusion through the biofilm (Costerton *et al.*, 1994, 1999). Additionally, it has been shown in a constant depth film fermentor (CDFF) model using a human saliva innoculum, that the addition of tetracycline at concentrations which could be found in the oral cavity alters the biofilm composition and enriches for tetracycline resistant bacteria (Ready *et al.*, 2002).

1.3.1.3 Antibiotic Resistance in the Oral Cavity

Antibiotic resistant bacteria are present in humans. Both infant and adult oral samples have been found to contain bacteria resistant to a number of antibiotics

(reviewed in Roberts, 1998a, 1998b). Even children who had not received antibiotics harboured resistant bacteria (Millar *et al.*, 2001; Ready *et al.*, 2003) with up to 100% of healthy child subjects under investigation harbouring ampicillin and erythromycin resistant bacteria in their oral cavity (penicillin and tetracycline resistant bacteria were present in 97% of the children) (Ready *et al.*, 2003). A further study put the prevalence of tetracycline resistant bacteria in the oral cavity of healthy children at 98% (Lancaster *et al.*, 2003, 2005). In the first study, 28% of isolates recovered exhibited resistance to two or more antibiotics illustrating the occurrence of multidrug resistant strains in the commensal flora (Ready *et al.*, 2003).

Tetracycline resistance is also common in the adult oral microbiota occurring in up to 11% of the total cultivable microbiota (Villedieu *et al.*, 2003). *tet*(M) is consistently identified as being the most common determinant (Lacroix & Walker, 1995; Villdieu *et al.*, 2003), it has been reported in *Streptomyces*, *Actinomyces*, *Veillonella*, *Streptococcus*, *Neisseria*, *Staphylococcus* and *Prevotella* oral isolates (Roberts, 1996), and on a number of different Tn*916*-like elements in the oral cavity (Bentorcha *et al.*, 1992). Additionally, *tet*(W), *tet*(O) and *tet*(Q) are also common (Olsvik *et al.*, 1994, 1995; Okamoto *et al.*, 2001; Villedieu *et al.*, 2003).

It must be noted that resistances to antibiotics (kanamycin, ampicillin and chloramphenicol) existed in oral bacteria prior to the clinical use of antibiotics, although they were only present as a very small proportion of the cultivable organisms and were low-level resistance compared to contemporary strains (Hughes & Datta, 1983; Houndt & Ochman, 2000). This illustrates that antibiotic-resistant bacteria have not only evolved due to their exposure to drugs, but

probably due to incidental transfer from antibiotic producing bacteria which harbour them as self-defence mechanisms (Edlund & Nord, 2000; Fine *et al.*, 1998).

1.3.1.4 Gene Transfer in the Oral Cavity

There is evidence that all three of the mechanisms of gene transfer described above occur in the commensal bacteria of the human oral cavity. Furthermore, it is likely that resistance genes can also be passed from the transient organisms to the resident microbiota and vice versa (Roberts *et al.*, 1999b; Eaton & Gasson, 2001).

DNA released from bacteria (either orally derived or from ingested food), including resistance-encoding sequences have the potential to transform naturally competent oral bacteria. Streptococci constitute ~ 20% of the normal flora and the genes involved in natural competence have been found in many of the species of the mitis and *S. angiosus* groups (Kolenbrander, 2000). Furthermore, filter sterilised saliva has been found to induce competence in the early coloniser *S. gordonii* DL1 (Mercer *et al.*, 1999). Both broth cultures and artificial biofilms containing *S. gordonii* were shown to transform with the erythromycin resistance plasmid pKMR4PE (Wang *et al.*, 2002) In addition, some *Neisseria* spp. and *Actinomyces* spp. are also naturally competent (Sun *et al.*, 2005; Tompkins *et al.*, 1997); and the biofilm mode of growth promotes increased rates of transformation as there is increased availability of DNA from dead cells held within the extracellular matrix in plaque which contributes to increased transformation rates (Li *et al.*, 2002).

Many organisms such as the black pigmented oral *Bacteroides* spp. have been shown to have the ability to transfer conjugal elements encoding tetracycline resistance in vitro (Guiney & Hasegawa, 1992); and the transfer of Tn*5397* from *B. subtilis* to *S. acidominimus* has been illustrated in a CDFF system (Roberts *et al.*, 1999a). Furthermore, Tn*916*-like elements are commonly found in oral isolates and have been shown to transfer tetracycline resistance determinants in model oral biofilms (Roberts *et al.*, 2001a). Further evidence for HGT comes from the high sequence similarities of the *mef* gene from viridans group streptococci which has been transferred to *E. faecium* and between *Streptococcus* spp. all of which share the upper respiratory tract as a habitat (Luna *et al.*, 1999). It is likely that conjugation is a major mechanism of gene transfer among bacteria of the oral cavity.

No studies of transduction in the oral cavity have been performed. However, in vitro studies have demonstrated the ability of various streptococci to receive genes by transduction (Mercenier *et al.*, 1988). Additionally bacteriophage have been isolated from the oral cavity (Hitch *et al.*, 2004) and many of the recently sequenced oral bacterial genomes have been shown to contain prophages, therefore, it is probable that this mechanism of gene transfer may occur in the GI tract.

1.3.2 The Gut/Faecal Microbiota

It is difficult to find a clear distinction between the gut and faecal microbiota. Many papers refer to investigations they have performed as looking at the 'gut microflora', however, the materials and methods of such studies would suggest 'faecal microbiota' would have been a more correct description since the samples used are not taken directly from the GI tract, but are faecal samples (Shimizu *et al.*, 2006; Tjellstrom *et al.*, 2005; Parracho *et al.*, 2005; Park *et al.*, 2005). However, the two are undoubtedly linked. The following description of the faecal microbiota includes any studies which use faecal samples but refer to their work as being on the gut or colon.

Bacteria represent a high proportion of faecal mass, accounting for approximately 54.7% of the total solids (Stephen & Cumming, 1980). The bacterial load of the intestine of a healthy adult is thought to be 1-1.5 kg (Owehand, 2002).

At present, the description of the human gut/faecal microbiota is limited by the inability to isolate and cultivate these organisms, and the lack of metagenomic studies on this environment. It is thought to consist of between 500-1000 species whose collective genomes are estimated to contain one hundred times the number of genes in the human genome (Backhed *et al.*, 2004; Blaut *et al.*, 2002). Estimates based on difference in diversity between culture studies and 16S rRNA library studies suggest that up to 80% of the microbiota is uncultivable (Duncan, 2003; Hayashi *et al.*, 2002; Suau *et al.*, 1999).

1.3.2.1 Colonisation of and Bacterial Diversity in the Gut

In humans, the gut is the natural habitat for a large and dynamic bacterial community (Table 1.9). As with the oral microbiota, colonisation begins just after birth with organisms from the environment (Fanaro *et al.*, 2003), the maternal intestinal flora (Fanaro *et al.*, 2003) and food borne bacteria (Martin *et al.*, 2003; Edwards & Parrett, 2002) and the development of the normal commensal flora proceeds over time (Kirjavainen & Gibson, 1999). Its composition is dependent upon a number of factors including the maternal microbiota, environmental (stress and dietary changes) and host genetic factors (Kirjavainen & Gibson, 1999), and it is host-specific (Dick *et al.*, 2005; Eckburg *et al.*, 2005). Studies with germ-free models have shown that the inhibition of the systemic response to commensal bacteria rapidly arises after initial colonisation, and that the development of important gut defenses such as the synthesis and secretion of polymeric immunoglobulin A and the generation of a balanced T helper cell response are dependent on colonisation with a diverse microbiota (Bourlioux *et al.*, 2003; Castagliuolo *et al.*, 1999; Shi & Walker, 2004).

Part of GIT	Common bacterial residents
Stomach	*Streptococcus* spp., Prevotella spp., Actinobacteria spp., Deinococcus spp., *Staphylococcus* spp., *Lactobacillus, Peptostreptococcus, H. pylori, Fusobacteria, Proteobacteria, Firmicutes, Fusobacteria* and *Deferribacteres.*
Small intestine	*Lactobacillus* spp., *Bacteroides* spp., *Clostridium* spp., *Mycobacterium* spp., Enterococci, bacteria of Enterobacteriaceae
Large intestine (colon)	*Bacteroides* spp., *Fusobacterium* spp., *Clostridium* spp., *Peptostreptococcus* spp., *E. coli, Klebsiella* spp., *Proteus* spp., *Lactobacillus* spp., Enterococci, *Streptococcus* spp., *Pseudomonas* spp., *Acinetobacter* spp., coagulase-negative Stapylococci, *S. aureus, Mycobacterium* spp., *Actinomyces* spp., *Bifidobacterium bifidum, Enterobacter* spp., *Peptococcus* spp., *Methanogens* (Archaea), *Salmonella* spp., *Ruminococcus* spp., *Eubacterium* spp.

Table 1.9: Common resident gut bacteria (taken from Upreti, 2004 with up-dates from Bik *et al.*, 2006).

The gut can be divided into three principle regions: the stomach, the intestines and the colon.

The stomach has a low microbial count of approximately ~100 CFU per ml since the environment has an inhibitory low pH (Rastall, 2004). Due to this only 29 out of 500 species found in the oral cavity have been recovered from faecal samples (Moore, 1994). Facultative anaerobes predominant (Bik *et al.*, 2006).

The small intestine also harbours a high degree of facultative anaerobes. In addition to lactobacilli and streptococci, enterobacteria are present in large numbers, although the anaerobes *Bifidobacterium*, *Bacteroides* and Clostridia are thought to predominate with CFUs of ~10^4 - 10^8 per ml (Rastall, 2004).

The colon is the most widely studied region of the GI tract and is most associated with the 'faecal microbiota'. It is the most heavily colonised region with a total population of between 10^{11} – 10^{12} CFU/ml, based on 16S rRNA studies (Rastall, 2004; Eckberg *et al.*, 2005).

The predominant genera in the human colonic microbiota are *Bacteroides* and *Prevotella* (which commonly occur at numbers in excess of $\log^{10}$ 9.5 cfu/g wet faeces), *Bifidobacterium*, *Eubacterium*, *Clostridium*, *Peptococcus* and *Peptostreptococcus* (Dick *et al.*, 2005; Rastall, 2004; Blaut *et al.*, 2002). Aerobic species (facultative anaerobes) are also present, including *Escherichia*, *Enterobacter*, *Enterococcus*, *Klebsiella*, *Lactobacillus* and *Proteus* (Upreti, 2004; Dick *et al.*, 2005). Studies have put the ratio of obligate anaerobes to facultative anaerobes at 300:1 (Duncan *et al.*, 2003). *E. coli*, the most common facultative anaerobe is thought to account for only 0.1% of the total bacteria (Blaut *et al.*, 2002). In addition to the bacteria, *Candida* and the protozoa *Trichomonas hominis*,

Entamoeba hartmanni, *Endolimax nana* and *Iodamoeba butschlii* are also common inhabitants of the large intestine (Duncan *et al.*, 2003).

It is has been found that bacteriophage also influence the diversity and population structure of the intestinal microbiota (Breitbart *et al.*, 2003). Molecular methods used to describe the microbiota have uncovered phages that infect *E. coli*, *Salmonella sp* and *B. fragalis* at up to 10^5 phages per g dry faeces (Breitbart *et al.*, 2003). The most common phage in a faecal sample from a healthy adult were bacteriophage Ah8 of *Listeria monocytogenes*, bacteriophage E125 of *Burkholderia thailandensis* and bacteriophage bIL285 of *L. lactis*. Overall it is estimated that there are approximately 2-5 times as many viral genotypes as the number of bacterial species in the human intestine, with a shot gun sequencing approach on a faecal viral library finding that ~59% of clones harboured inserts with no significant similarity to anything in the database (Breitbart *et al.*, 2003).

1.3.2.2 Antibiotic Resistance in the Gut

The GI tract provides huge potential for genetic exchange due to its dense, diverse flora and the input of transient organisms and DNA from exogenous sources (Upreti *et al.*, 2004). Only recently have resistance genes and their associated mobile elements been looked for in the human GI tract. Both novel genes and CTns have been described (Scott, 2002; Calva *et al.*, 1996; Osterblad *et al.*, 2000; Rice *et al.*, 2004; Shoemaker *et al.*, 1992).

Studies by Houndt and Ochman, (2000) have shown that among strains of enteric bacteria isolated from diverse hosts between 1885-1941 before the commercial

application of antibiotics began, levels of resistance were low. This is presumably due to the lack of a strong selective pressure. Strains from collections originating from 1972-1982 showed an increase in high-level resistance in 20% of isolates. However, Corpet (1992) reviewed short-term studies on whether antibiotic residues can modify the human gut flora and found that volunteers given low doses of ampicillin or oxytetracycline (1.5 mg/day and 2.0 mg/day respectively) showed no significant changes in the numbers of resistant faecal enterobacteria (Corpet, 1992); and De La Cochotiere *et al.*, (2005) found that after a course of amoxicillin (1.5 g/day) volunteers' faecal microbial diversity returned to pre-antibiotic state after 60 days.

However, tetracycline resistance has been found in up to 12 % of infantile colonic *E. coli*, with *tet*(A) and *tet*(B) occurring in 49 % and 51 % of tetracycline resistant isolates, respectively (Karami *et al.*, 2006). Colonisation with tetracycline resistant strains was unrelated to treatment with antibiotics indicating there is limited pressure against the carriage of *tet* genes in the gut microbiota (Karami *et al.*, 2006). In other studies tetracycline resistance has been found in 32.8 % of faecal bacteria from individuals not receiving antibiotic therapy (Stark *et al.*, 1993); and in 30 % of *Bifidobacterium* spp. species from the human GI tract (Delgado *et al.*, 2005). Additionally, resistance to ampicillin, cefoxitin and cefuroxime has been found in bacteria from 70 % of faecal samples from hospitalised patients (mainly *Bacteroides thetaiotaomicron*, *Clostridium innocuum* and *Bacteroides ovatus*) (Stark *et al.*, 1993).

In addition, in the last ten years, the GI pathogens *Salmonella* spp., *Campylobacter* spp. and *H. pylori* have become resistant to antibiotics commonly used to treat

them (Threlfall, 1999; Giraud *et al.*, 2006; Keller & Perreten, 2006). Fluoroquinolones and third generation cephalosporins are no longer useful against salmonellae, fluorquinolones and macrolides are redundant in the treatment of *Campylobacter* infections and metronidazole and clarithromycin are no longer effective against *H. Pylori* (Kim, 2006). Studies have suggested that the use of antibiotics in food animals selects for antibiotic resistance genes which are then transferred to humans on consumption (Krause, 2002).

1.3.2.3 Gene Transfer in the Gut

Horizontal gene transfer has not yet been studied *in situ* in the colon, however, various transfer experiments have been conducted that implement colonic species in such processes. Transfer of *vanA* has also been detected from a resistant animal derived *E. faecium* to a sensitive human derived *E. faecium* in human faecal flora-associated mice (Bourgeois-Nicolaos *et al.*, 2006); *tet*(M) has been shown to transfer from *Lactobacillus* spp. to *E. faecalis* (Gevers *et al.*,2003); the nonrecombinant plasmid pAMbeta1 has been observed to transfer from *L. lactis* strains to enterococci in a faecal flora-associated mouse model system (Tuohy *et al.*, 2002); and Tn*916* (Bahl *et al.*, 2004) and Tn*1549* (Launay *et al.*, 2006) have been shown to readily transfer between *E. faecalis* strains and from *Clostridium symbiosum* to *Enterococcus* spp., respectively in the gut of gnotobiotic mice. Futhermore, the carriage of *tet*(Q) alleles within the human GI bacteroides populations has increased from ~30% to more than ~80% of isolates over the past thirty years, all alleles except one showed between 96-100% identity, implicating HGT (Shoemaker *et al.*, 2001).

In addition, transfer events between human colonic bacteria and human oral anaerobes (potentially via an intermediate host) has been detected, with tetracycline resistance determinants of the human *Bacteroides uniformis* and the oral *Prevotella intermedia* being 100 % identical at nucleotide level (Shoemaker *et al.*, 1991; Nikolich *et al.*, 1994a). Furthermore, *tet*(Q) of the ruminal *Prevotella ruminocola* is almost identical to (> 97 %) the *tet*(Q) of human *Bacteroides* strains, implicating HGT between these species and possible intermediate hosts (Nikolich *et al.*, 1994b). Similar high identities have been observed for *tet*(W) genes in the ruminal bacteria *B. fibrisolvens*, *Selenomonas ruminantium* and *Mitsuokella multiacidus* (Barbosa *et al.*, 1999); sequences flanking the *tet*(W) in *Butyrivibrio fibrisolvens* isolates and other gut genera were found to be between 96 – 100 % similar in all strains examined, with Tn*B1230* implicated (Kazimierczak *et al.* 2006).

There is no evidence of transduction or transformation occurring in the gut. However, the recent investigation of the faecal viral metagenome (Breitbart *et al.*, 2002) has illustrated the huge number of phage present, and thus the potential for transduction to occur. This study found Siphophages to be the most common of the recognisable bacteriophages, these have previously been implicated in transduction (Seguritan *et al.*, 2003). Furthermore, chloramphenicol resistance has been shown to transfer by phage among *E. coli* strains in the house fly gut (Petridis *et al.*, 2006), tetracycline resistance transfer between *S. aureus* strains has been shown to be phage mediated (Perira *et al.*, 1997) and *mef*(A) in *S. pyogenes* have been shown to be prophage-associated (Giovanetti *et al.*, 2005).

Due to the enormous amounts of bacteria (both live and dead) that pass through the GI tract and the lysis of these bacteria by stomach acid and intestinal enzymes (Drouault *et al.*, 1999), there is likely to be DNA available for uptake via transformation (although this has yet to be conclusively proven). Furthermore, the dense population of bacteria in the colon provides favourable conditions for DNA uptake (Salyers *et al.*, 2004).

1.4 Metagenomic Studies

1.4.1 The Concept of Metagenomics

Microorganisms account for a huge proportion of life on earth.

Bacteria were first reported in 1663 by Antonie van Leeuwenhoek (reviewed in Tan, 2003). Work on the microbial world continued through culture studies thus unintentionally dividing it into the cultured and uncultured bacteria. However, it is only relatively recently that the extent of the uncultured world has been recognised (Figure 1.11). DNA-DNA reassociation studies have since demonstrated that the diversity of bacteria in the soil was ~100 fold greater than could be accounted for by culture techniques alone (Torsvik & Ovreas, 2002; Streit & Schmitz, 2004). This, and the realisation that much of the microbial world is the foundation for the Earth's geochemical cycles (reviewed in Streit & Schmitz, 2004) demonstrated the need to explore further the unknown. Thus 'metagenomics' was born. Metagenomics is defined as the culture-independent analysis of microbial communities or the 'compound genome of the environmental microbiota' (reviewed in Schloss & Handelsman, 2003; Steele & Streit, 2005), it is also termed 'environmental genomics' or 'community genomics' (reviewed in Rodriguez-Valera, 2004; Tyson & Banfield, 2005; Handelsaman, 2004).

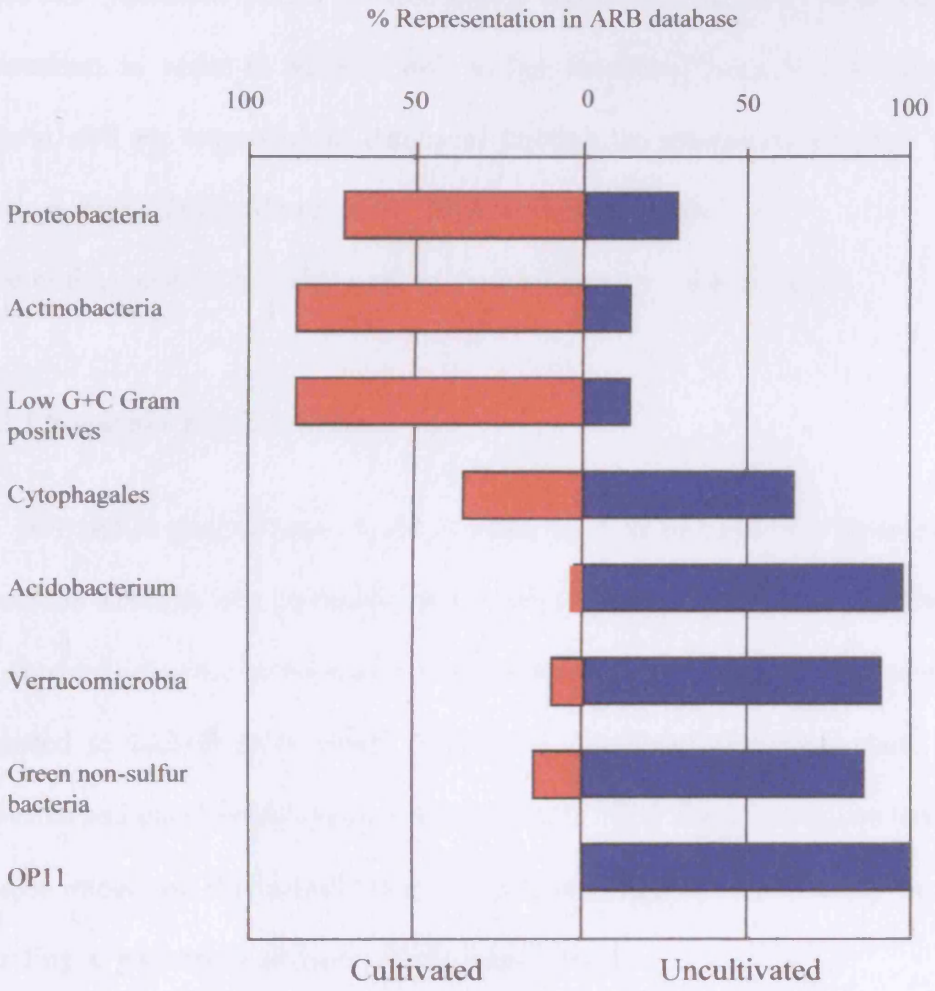


Figure 1.11: Relative representation in selected bacterial divisions of 16S rRNA sequences from cultivated and uncultivated organisms. Compiled from 5224 and 2918 sequences from cultivated and sequences from uncultivated organismss, respectively. (Taken from Hugenholtz *et al.*, 1998). Red sections indicate cultivable portions of each division, blue sections indicate uncultivable portions

1.4.2 The Focus of Metagenomic Studies

There has been a dramatic increase in the application of molecular approaches applied to microbiota based on 16S rRNA gene sequence, however, sequence data does not provide any information about the organisms phenotype (Steele & Streit, 2005; Hugenholtz *et al.*, 1998). Instead the emphasis has begun to shift into

'functional genomics' which is based on the comparison of nucleic acid sequence information in order to identify and assign functional roles to non-cultivable bacteria, and the expansion of databases through the sequencing of novel genes (Steele & Streit, 2005; Venter *et al.*, 2004; Gill *et al.*, 2006).

Some of the common methods used in these studies are outlined below.

1.4.2.1 Sequence Based Studies

The 16S rRNA gene (Figure 1.12) is often used to describe the diversity and population structure of a particular environment (Schloss & Handelsman, 2004). It is a standard reference in the identification of bacteria and the databases have been expanded to include over 90000 16S rRNA sequences, including those from cultivated and uncultivated organisms (Clarridge, 2004). Such techniques have had a major impact on the classification of bacteria which were previously grouped according to phenotypic properties (Clarridge, 2004).

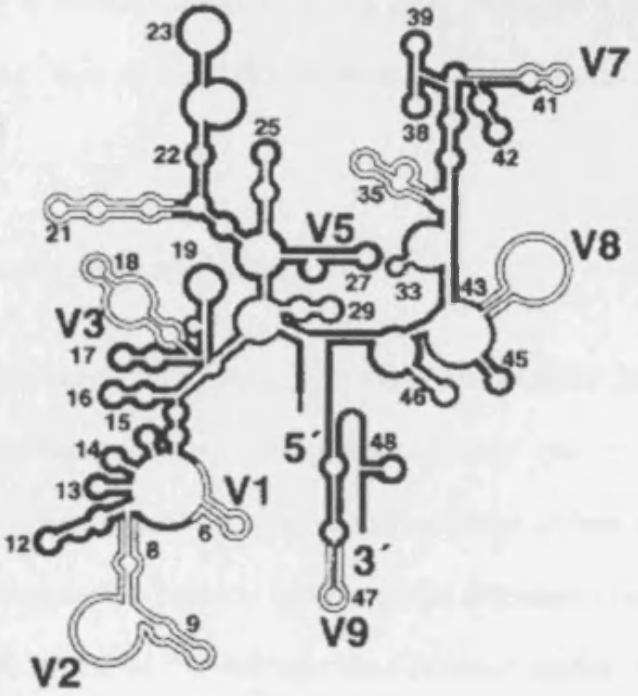


Figure 1.12: Genetic organisation of the 16S rRNA gene. The rate of change in the gene is not known, but it has been determined to represent evolutionary distance and relatedness of organisms (Ochman *et al.*, 2000). The 16S rRNA gene sequence is ~1550 bp and contains both variable (double lines) and conserved (thick black lines) regions. Universal primers used to amplify sequence for comparison are based either side of the variable regions V1 or V3 (image taken from Tortoli, 2003; Klijn et al., 1991).

Since the use of the 16S rRNA gene as a phylogenetic tool, 50 bacterial phyla have been identified. Half of these are exclusively composed of uncultured bacteria illustrating the extent to which molecular techniques have expanded our knowledge (Schloss and Handelsman, 2004). However, in 2004 there were still a predicted 10^7-10^8 different species of bacteria still to be discovered, a Figure based on bacterial rarefaction curve analysis which is used to estimate the completeness of data sampling (Hugenholtz *et al.*, 2002).

Importantly, HGT appears to affect the informational genes (those involved in transcription and translation) far less than it does the operational genes (those

involved in housekeeping), allowing us to disregard it as a method of obscuring phylogeny (Jain *et al.*, 1999; Schloss & Handelsman, 2004; Hugenholtz *et al.*, 2002).

1.4.2.2 Analyses of Microbiota

Techniques used to investigate environmental samples focus either on description and quantification of species present (mainly due to the abundance of 16S sequence data available) or the in situ activities of bacteria (including numerous as-yet-unknown biochemical and metabolic activities) (Table 1.12) (Spiegelman *et al.*, 2005). Some of the methods used in these studies, and their limitations are outlined in Table 1.10.

Method	Uses	Limitations
Cultivation	Isolation, 'the ideal'	Not representative, slow and laborious
16S rDNA Sequencing / 16S rRNA clone libraries	Phylogenetic identification	Laborious, subject to PCR / cloning biases
DGGE/TGGE/TTGE	Monitoring of community shifts; rapid comparative analysis	Subject to PCR biases and co-migration of bands (disorting diversity); semi-quantitiative; identification requires clone library
T-RFLP	Monitoring of community shifts; rapid comparative analysis; very sensitive; potential for high throughput	Subject to PCR biases; semi-quantitiative; identification requires clone library
SSCP	Monitoring of community shifts; rapid comparative analysis	Subject to PCR biases; semi-quantitative; identification requires clone library
RT (real time)-PCR	Quantification of bacteria in an environmental sample. Promising for species present at low concentrations	Requires sequence information
FISH	Detection; enumeration; comparative analysis possible with automation. In combination with flow cytometry can sort uncultivable from cultivables to access to sequence information	Requires sequence information; laborious at species level; high detection levels; relies on cell permeability
Dot-blot Hybridisation	Detection; estimates relative abundance	Requires sequence information; laborious at species level
Quatitative PCR	Detection; estimates relative abundance	Laborious
Diversity Microarrays	Detection; estimates relative abundance	In early stages of development; expensive
Shotgun libraries	Access to sequence information of the whole metegenome	Subject to cloning biases; high detection level; generates sequences encoding unknown functions
Non-16S rRNA profiling	Monitoring of community shifts; rapid comparative analysis	Identification requires additional 16S rRNA-based approaches

Table 1.10: A summary of current techniques used to study complex microbial ecosystems (adapted from Zoetendal *et al.*, 2004; additional information from Gafan *et al.*, 2005).

1.4.2.3 Metagenomic Libraries

One method for accessing the functional information of a microbiota that eliminates this PCR-induced bias and the need for sequence information is the construction of metagenomic libraries constructed from the total DNA extracted

from an environmental sample (although this method has biases of its own) (Rondon *et al.*, 2000; Handelsman, 2004). In 2000, Rondon *et al.*, published a landmark paper describing the use of Bacterial Artificial Chromosomes (BACs) to clone total community DNA thus accessing the entire metagenome, potentially allowing the unbiased cloning of intact genes with all the long range controlling elements that drive expression and even entire biosynthetic/metabolic pathways. It is one of the most effective techniques for identifying functional genes from environmental samples (Entcheva *et al.*, 2001).

As with 16S libraries a number of vectors can be used. In recent years the Bacterial Artificial Chromosome (BAC) has been a popular choice (Beja *et al.*, 2000; MacNeil *et al.*, 2001; Gillespie *et al.*, 2002; Riesenfeld *et al.*, 2004).

1.4.2.3.1 Bacterial Artificial Chromosomes

BACs were developed by Shizuya and colleagues in 1992 as a bacterial cloning system for mapping and analysis of complex genomes as part of the effort to create a high-resolution map of the human genome. The BAC (Figure 1.13) is based around the mini-F plasmid pMB0131 which contains genes that regulate its own replication (*OriS* and *repE*) and control its copy number (*par* operon, which is involved in the exclusion of multiple f factors (Easter *et al.*, 1998) thus allowing the stable maintainence of inserted bacterial DNA. The addition of a chloramphenicol resistance gene as a selectable marker; a cloning segment composed of *cosN* sites from bacteriophage λ and *loxP* sites from P1, and a multiple cloning site flanked by promoters completed the vector (Figure 1.13) (Shizuya *et al.*, 1992; 2001). The vector was used to clone inserts of more than 300 kb (Shizuya *et al.*, 1992)

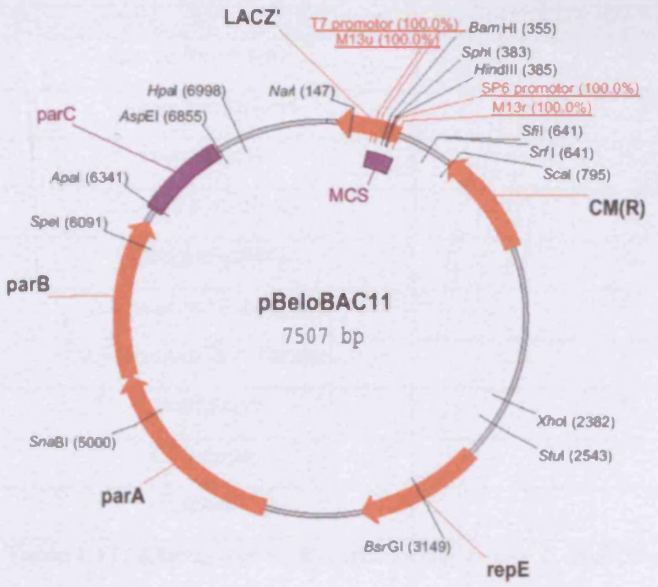


Figure 1. 13: pBeloBAC11, derived from Shizuya's original BAC vector. Orange arrows and purple box labelled *parC* indicate genes, purple box beneath lacZ represents the multiple cloning site (www.rzpd.de)

1.4.2.3.2 Current Applications of the BAC System

1.4.2.3.2.1 Single species studies

Perhaps the most useful study based around a single species library is that analysing the expression of *B. cereus* genes cloned into BAC in an *E. coli* host. It high-lighted the need for continued development of molecular tools. Rondon *et al.*, 1999 screened the BAC library for expression of *B. cereus* activities and found that 60% were detectable in the Gram negative host (Table 1.11).

Activity Tested	Expression Detected
Starch hydrolysis	
Casein hydrolysis	
Haemolysis	+
Esculin hydrolysis	+
Orange pigment	+
Ampicillin resistance	+
Zwittermicin A resistance	+
Lecithinase	+
Chitinase	
Lipase	

Table 1.11: Expression of *B. cereus* activities in *E. coli* (taken from M Rondon *et al.*, 1999).

1.4.2.3.2.2 Metagenomic studies

Metagenomic studies are capable of identifying novel bacterial properties. To date, most studies have concentrated on aquatic and soil environments using a variety of different vectors (Table 1.12).

Vector	Metagenome/ sample	Novel Genes/ Characterizations	Reference
BAC	Marine	Novel bacteriorhodopsin	Beja *et al.*, 2000a, 2000b
BAC	Soil	Kanamycin resistance encoded by a novel aminoglycoside gene	Riesenfeld *et al.*, 2004
Small insert (pJN105 / pCF430)	Soil	Eight novel aminoglycoside resistance genes and a novel tetracycline resistance gene	Riesenfeld *et al.*, 2004
BAC	Soil	Novel indirubis-like antimicrobial compound	MacNeil *et al.*, 2001
BAC	Soil	Turbomycin A and B synthesis characterisation	Gillespie *et al.*, 2002
Fosmid	Soil	Novel copper-containing nitrate reductase	Treusch *et al.*, 2005
Cosmid	Soil	Novel Violacein-like antibacterial pigment	August *et al.*, 2000
Cosmid	Soil	Putative stereoselective amidase, 13 cellulases, α-amylase, 1,4-α-glucan branching enzyme, 2 pectate lyases	Voget *et al.*, 2003
Cosmid	Soil	Long chain *N*-Acyltyrosine synthases	Brady *et al.*, 2004
pBluescriptSK+	Soil	4-hydroxybutyrate utilisation	Henne *et al.*, 1999
Fosmid	Marine	Characterisation of uncultured prokaryotes	Vergin *et al.* ,1998 Stein *et al.*, , 1996
Fosmid	Soil	Uncultured Crenarchaeote	Quaiser *et al.*, 2002
Fosmid	Thermal soil	Thermostable esterase	Rhee *et al.*, 2005
Fosmid	Marine	Novel candidate phylum Poribacteria; novel molybdenum containg oxidoreductas	Fieseler *et al.*, 2006
Fosmid	Marine	Discovery of a novel lineage of *Alvinella*	Moussard *et al.*, 2006
TOPO-XL	Human saliva/plaque	Tetracycline resistance (tet(37))	Diaz-Torres *et al.*, 2003

Table 1.12: Metagenomic libraries and the novel characteristics and / or genes found during their screening.

1.4.3 The Future of Metagenomics

In light of the findings by Rondon *et al.*, (1999) that only 60% of genes from a Gram positive organism are expressed in the Gram negative *E. coli* host a number of new vectors have been developed in order to provide a range of hosts to use when performing functional screens (Table 1. 13). Through construction of libraries in these vectors and their screening in alternative hosts, differences in the expression of certain traits have been detected (Alduina *et al.*, 2003; Martinez *et al.*, 2004; Wang *et al.*, 2000).

Vector	Hosts	Important Characteristics	Reference
BIBAC (binary bacterial artificial chromosome) & TAC (transformation-competent BAC)	*E. coli* and various plants	Transforms plants directly	Liu *et al.*, 2002
ESAC (*E. coli* – Streptomyces Artificial Chromosome)	*E. coli* *Streptomyces coelicolor*	BAC-based shuttle vector. Incorporates into Streptomyces host using ΦC31 attP-int system	Sosio *et al.*, 2000, 2001 Alduina *et al.*, 2003
pMBD7, -9, -12	*E. coli* *Sm. lividans* *Pseudomonas putida*	BAC based shuttle vector	Courtois *et al.*, 2003
pDL276, pDL278	*E. coli* *B. subtilis* *E. faecalis* Streptococci	Produces random, representative libraries with inserts of 10 Kb	Dunny *et al.*, 1991

Table 1. 13: Alternative vectors for metagenomic cloning with both Gram positive/eukaryotic and Gram negative hosts.

1.4.4 Analysis of Metagenomic Data

Since the rise in the use of BACs and other HMW-insert vectors to create libraries, the development of supporting analytical techniques is required in order to

optimise the benefits of these systems (Chen & Pachter, 2005; Ball & Trevors, 2002).

Libraries have been screened for beneficial genes either by functional or sequence screens (Handelsman, 2004). Both have advantages. Sequence-based approaches allow the quick identification of genes through comparisons to the databases, and if a 16S rRNA or other marker gene is identified in a clone then the phylogenetic identity of the insert can be determined (Handelsman, 2004; Francke *et al.*, 2005). Functional screens are laborious and time consuming (although pooling schemes have been described) (Bruno *et al.*, 1995) but offer an opportunity to discover functional, novel genes if appropriate assays exists (Yun *et al.*, 2005).

The ultimate aim of sequenced-based approaches is to assemble whole genomes of uncultured organisms (Francke *et al.*, 2005). Liles *et al.*, (2003) have so far assembled ~3 Mb of *Acidobacterium* DNA from 73 metagenomic clones. A similar project is also underway for an uncultivated soil Crenarchaeote (Quaiser *et al.*, 2002). However, at present this approach is largely based around anchors such as 16S rRNA or *recA* genes.

So far, fewer products have been identified than expected (products found to date are listed in Table 1.12). One explanation is that the synthesis of some products often requires large fragments of DNA of up to 100 genes to be cloned (Shleh-Lakha *et al.*, 2005). This is only likely to occur rarely in a library. One way around this is the development of highly sensitive assays which are capable of detecting low levels of activity (Williamson *et al.*, 2005; Schloss and Handelsman, 2004), such as Substrate-Induced Gene-Expression Screening (SIGEX) which selects

clones harbouring catabolic genes with fluorescence activated cell sorting by using tagged aromatic hydrocarbons (Yun & Ryu, 2005)

1.4.4.1 Combined Approaches

The combined use of library construction and array analysis of environmental samples allows clones of interest to be identified without the need for expression in a heterologous host and without laborious screening of individual clones (Ball, 2002). Sebat and colleagues, (2003) developed an array consisting of DNA from previous isolates, reference strains and whole community DNA for screening a ground water cosmid library. This produced a comparative genomic hybridisation profile for each cloned insert. Furthermore, clones which failed to hybridise to anything were easily identified for sequencing. This approach identified functions with potential ecological importance including those involved in hydrogen oxidation, nitrate reduction and transposition. The study recognized the potential use of such arrays for rapid screening of metagenomic libraries.

Call *et al.* (2003) have since developed a glass-based microarray for detection of 17 tetracycline resistance determinants (*tet*(A)-(E), *tet*(G), *tet*(H), *tet*(L), *tet*(M), *tet*(O), *tet*(S), *tet*(W), *tet*(Z), *tet*(30), *tet*A(P), *tet*B(P), and *otr*(B)0, bla_{TEM-1} and 16S rRNA oligonucleotides) which could be exploited for detection of resistance genes in environmental libraries.

1.4.5 Molecular Techniques Applied to the GI Tract

Many of the techniques outlined above have been employed to further our understanding of the oral and intestinal microbiota (Table 1.14). Currently most of

the work focuses on descriptions of the diversity in both health and disease (O'Sullivan, 2000; Gill *et al.*, 2006). However, more recent work has focused on functional screens and COG (Clusters of Orthologous Groups) analysis (which groups functionally related genes using evolutionary relationships and thus estimates community richness of metabolic functional groups in a microbiota) (Tannock, 2001, 2002; Diaz-Torres *et al.*, 2002), to gain a more meaningful insight into the microbiota.

Method	Sample	Study	Reference
FISH TGGE	Faecal	Identification of the predominant bacteria species, and investigation of changes in diversity over time	Zoetendal *et al.*, 2004
TGGE	Faecal	Demonstration that the intestinal flora is host specific, indicating the role of genetic factors in colonisation	McCartney, 2002
16S rDNA libraries/PCR	Various along the GI tract	Demonstration of the dominance of 6 phyla: firmicutes, Proteobacteria, Bacteroides, Fusobacteria, Verrucomicrobia, and the Actinobacteria. Demonstrated Clostridia and Streptococcus-like species predominate in the colon Demonstration that Firmicutes and Actinobacteria are the only divisions represented in the distal gut, and Methanobrevibacter smithii is the only archael phylotype	X Wang *et al.*, 2003 M Wang *et al.*, 2005 Vaahtovuo *et al.*, 2005 R Wang *et al.*, 1996 Wilson & Blitchington, 1996 Gill *et al.*, 2006
16S rDNA + RFLP analysis	Jejunal and ileal mucosa	Measured diversity in elderly patients	Hayashi *et al.*, 2002, 2003, 2005 Jernberg *et al.*, 2005
SSCP fingerprinting RT-PCR	Faecal	Investigated diversity in the colonic microbiota of patients with inflammatory bowel disease (demonstrated a reduction in diversity of 50% for Crohn's patients and 30% for UC patients)	Ott *et al.*, 2003
16S rDNA libraries	Saliva/ plaque	Measured diversity in healthy subjects, endodontic patients and those with aggressive periodontitis	Saito *et al.*, 2006 Sakamoto *et al.*, 2000, 2004
T-RFLP	Saliva/ plaque	Assessment of oral microbial diversity	Sakamoto *et al.*, 2003, 2005
DGGE	Saliva/ plaque	Investigation of diversity over time, between individuals and in dental plaque microcosms	Rasiah *et al.*, 2005 Y Li *et al.*, 2005
FISH	Saliva	Indentification and quantification of bacteria in carious dentine	Banerjee *et al.*, 2002
TOPO-XL metagenomic libraries	Saliva/ plaque	Screening of the oral microbiota for tetracycline resistance	Diaz-Torres *et al.*, 2003

RT-PCR	Plaque	Quantification of periodontopathic bacteria	Sakamoto *et al.*, 2004
RT-PCR	Faeces	Characterisation of faecal flora and investigation of the effects of antibiotic treatment	Bartosch *et al.*, 2004.
Whole-genome Shotgun Sequencing	Faeces	Investigation of the diversity of metabolic functions in the faecal microbiome – demonstration of enrichment in genes involved in metabolism of complex carbohydrates which the human genome lacks	Gill *et al.*, 2006

Table 1. 14: Metagenomic studies of the Human GI Tract.

Despite all the advances in this field outlined above, the concept of finding a certain gene or pathway in an environmental sample using metagenomics depends on a number of factors. The abundance of the gene in the environmental sample; the length of the gene or operon; the size of insert and the presence of expression signals that are functional in the host organism; and the correct folding of the resulting protein in a heterologous host by trans acting host factors (chaperones, cofactors, protein-modifying enzymes) all contribute to the likelihood of successful cloning (Gabor *et al.*, 2004).

The minimal requirements for gene expression are an appropriately spaced promoter for transcription, a ribosome binding site (RBS) and a start codon for initiation of translation, both of which need to be compatible with host transcription factors (Gabor *et al.*, 2004).

In metagenomic cloning, expression may be via

i) independent gene expression (both the RBS and promoter are provided by the insert)

ii) expression as a transcriptional fusion with only the RBS located on the insert

iii) as a translational fusion (the RBS and promoter are provided by the vector), although this is highly unlikely to occur.

Gabor *et al.*, (2004) have taken these factors into account and designed to statistical models to determine what proportion of genes might be expressed in an *E. coli* host, taking into account the three modes of gene expression listed above. Their results how approximately 40% of enzyme activities from a metagenome can be expected to express in *E. coli* and thus recovered by random cloning. Therefore, the challenge still remains to access all the genetic information from a metagenome.

Perhaps one concern of the wide use of these methods to define environmental microbial diversity is the deposition of sequences in the database which provide no further information than the degree of relatedness of 'clones' to cultivable species. While it allows us to numerically quantify diversity, these methods shed no further light on the role of these novel species in the microbiota. There is also concern that the relative ease of obtaining Mb of sequence data is eclipsing the need to continue culture based studies, especially in light of the fact that novel cultivation stratergies are possible and have recently been developed for butyrate-producing (Gourque-Jeannot *et al.*, 2006) and cellobiose-degrading bacteria (Zoetendal *et al.* 2003) allowing access to potentially marketable novel biochemical pathways, and a greater understanding of its role within the environment.

1.5 Rational for This Study

The human commensal microbiota is known to be a reservoir of antibiotic resistance genes (Lacroix & Walker, 1995; Millar *et al.*, 2001; Ready *et al.*, 2003; Villedieu *et al.* 2003). This has important impact on antimicrobial therapy (reviewed in Martinez & Baquero, 2002; Lipsitch & Samore, 2002). However, to date, studies have concentrated on which resistance determinants and genetic supports are present in the cultivable flora (Lacroix & Walker, 1995; Millar *et al.*, 2001; Ready *et al.*, 2003; Villedieu *et al.* 2003). Since ~50 % of the oral microbiota (Wade *et al.*, 2002); and ~80 % of the faecal microbiota (Hayashi *et al.*, 2002; Duncan *et al.*, 2003) is yet to be cultivated, the presence of some resistance determinants may be inaccurately estimated. Indeed it has been shown that the uncultured portion of the oral microbiota harbours novel tetracycline resistance determinants (Diaz-Torres *et al.*, 2003). Therefore, in order to provide effective antibiotic treatment for infections of the GI tract, an antibiotic resistance profile for the complete microbiota is required.

The aims of the study are:

1). To determine the incidence of resistance to the antibiotics erythromycin and tetracycline in the aerobic cultivable and total flora of the human oral and faecal microbiota of samples from six European countries.

Only Gram positive isolates from the culture study will be investigated further as they represent a distinct phylogenetic group which can be investigated in detail. Furthermore, as this project was part of a larger European-wide study on the biology of Gram positive organisms, can be compared to the results obtained by

our European partners involved in the ARTRADI project (an EU project). Enough tetracycline resistant BAC clones will be identified so that a significant number of inserts from Gram positive organisms can be isolated for comparison with the Gram positive isolates in order to determine if there is a difference between the genetic basis of tetracycline resistance in the cultivable and uncultivated portions of the microbiota in that phylogenetic group.

2). To describe the genetic basis of tetracycline resistance in the Gram positive cultivable portion of the samples and compare it to the genetic basis of resistance in the whole microbiota.

3). To construct 12 metagenomic libraries, one from each of the oral and faecal samples obtained from each partner country.

4). To screen the metagenomic libraries for tetracycline resistance and characterise any novel genes or mobile genetic elements.

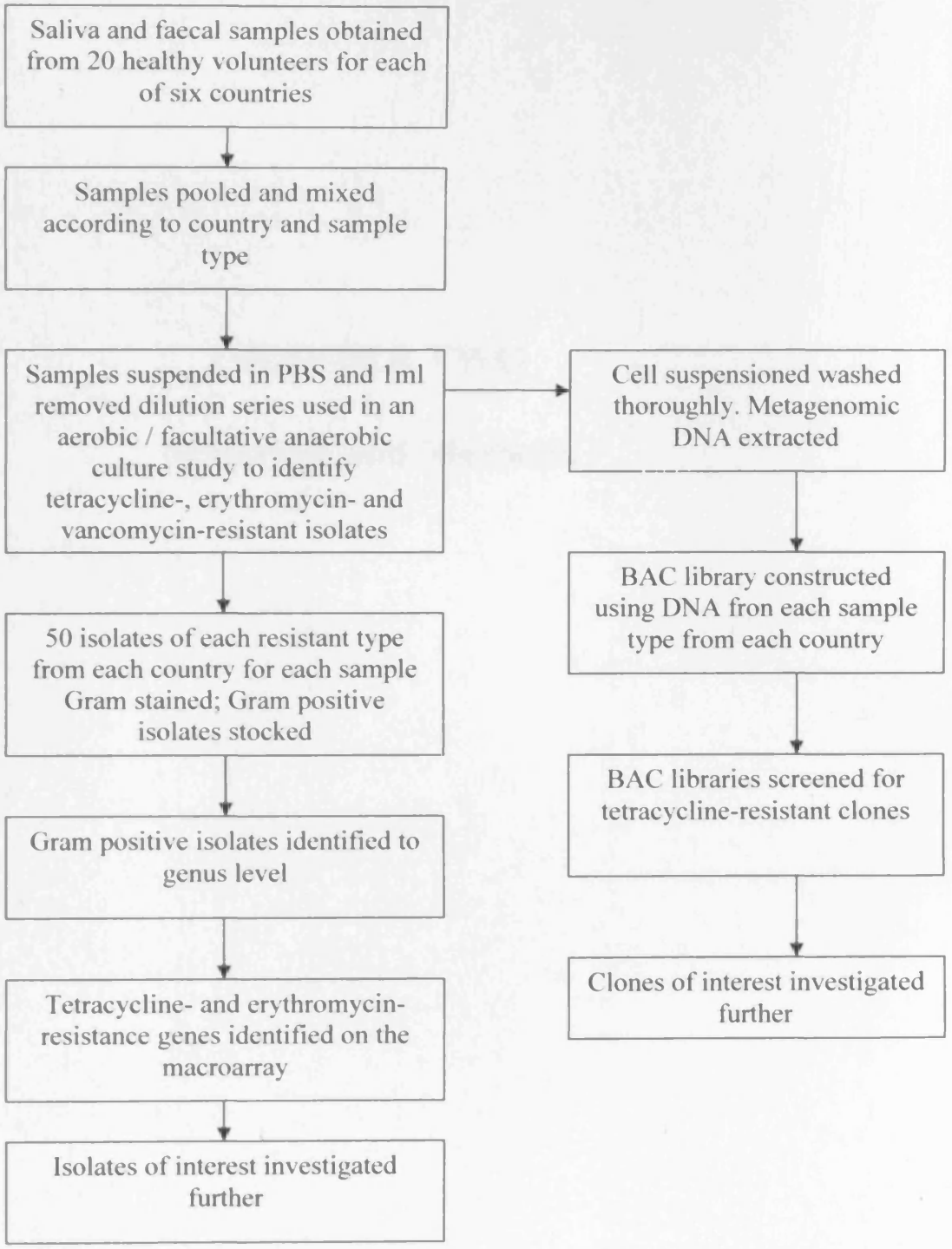


Figure 1.14: Flow chart illustrating path of investigation in this study.

CHAPTER TWO

Materials and Methods

2.1 Bacterial Strains and Plasmids

Bacterial strains and plasmids used in this study are listed in Table 2.1.

Strains and plasmids	Relevant characteristics/source	Reference
Strains		
S. aureus NCTC 6571	Antibiotic sensitive	www.hpa.org.uk
B. subtilis BS34A	*tet*(M), Tn*916*	Roberts *et al.*, 2001
E. coli::pGEM	*tet*(O)	Aminov *et al.*,2001
K10	*tet*(32)	Melville *et al.*, 2001
E. faecium 664:IHI	*tet*(S)	Roberts *et al.*, 2006
E. coli EPI 300	BAC host	Epicentre
E. coli DH10b	pUC19/pDL278 host	Invitrogen
S. mutans UA159	pDL278 host	Dunny *et al.*, 1991
Plasmids		
pUC19	Small insert cloning vector, ampicillin resistant	Invitrogen
pCC1BAC	Large insert cloning vector, chloramphenicol resistant	Epicentre
pDL278	Small insert shuttle vector, spectinomycin resistant	Dunny *et al.*, 1991

Table 2.1: Bacterial Strains and Plasmids used in this study.

2.2 Chemicals and Reagents

All growth media were obtained from Oxoid Ltd (Basingstoke, UK); All antibiotics and other chemicals were obtained from Sigma Aldrich (Poole, UK); All molecular biology enzymes were obtained from Promega (Southampton, UK); Competent cells

were obtained from Epicentre (distributed through Cambio, Cambridge, UK) unless otherwise stated. Composition of buffers and solutions are listed in Appendix 1.

2.3 Sampling and Processing of Saliva and Faecal Samples

2.3.1 Subjects

20 healthy adults who had not received antibiotic therapy in the previous three months were recruited as volunteers from each of the countries under investigation (England (Eastman Dental Institute, University College London), France, 2 centres (Faculté de Parmacie, Université Paris Sud and INRA-UEPSD, Domain de Vilvert), Finland (VTT Biotechnology), Italy, 2 centres (Istituto di Microbiologia, Universita di Ancona and Istituto Superiore di Sanita, Roma), Norway (IMB University of Tromsó) and Scotland (Rowett Research Institute). No set of 20 were exclusively male or female and represented a range of ages (Appendix 2)

2.3.2 Sample Collection

Instructions were given to each vounteer (Appendix 3). Saliva samples (~5ml) were acquired using 1g sterile low melting point W4 physiowax (Raymond A Lamb Ltd. Eastbourne, East Sussex) to stimulate saliva and dislodge plaque. Samples were collected by expectoration into a sterile container (care was taken not to contaminate the samples with bacteria from the lips and chin). An equal volume of Reduced Transport Fluid (RTF) (Syed *et al.*, 1972) was added to each sample in a microbiology safety cabinet (BioMAT-1 Class 1 microbiological safety cabinet) to avoid contamination. Faecal samples (>2.5g) were obtained from 20 individuals from the same centres. Volunteers defecated into a disposable paper container (Sarstedt, Leicester, UK). They then used a disposable spatula to transfer faeces into a sterile

plastic collection tube (Sarstedt) until it was a quarter full. Cary-Blair medium was then used to completely cover the sample in a safety cabinet so that the container was approximately half full.

Each set of samples was kept separate according to sample type (oral/faecal) and country of origin. They were processed with 48 h of their collection.

2.3.3 Processing of Saliva and Faecal Samples

2.3.3.1 Saliva Samples

The saliva was vortexed (Fischerbrand whirlimixer) for 30 s to resuspend any settled plaque or planktonic cells. All samples from individual countries were pooled in a sterile 200ml Duran (Fisher Scientific, Loughborough, UK).

2.3.3.2 Faecal Samples

2.5g of each of the 20 faecal samples from a country were pooled, mixed by manual kneading in a sterile disposable plastic bag (Steward Scientific) and divided into 5g aliquots. The faeces were suspended in 50ml PBS and centrifuged at 4000 rpm (2862 x g, eppendorf centrifuge 5804R) for 2 mins to pellet the coarse sediment. The supernatant was removed and centrifuged at 6000 x rpm (6800 x g, Eppendorf centrifuge 5804R) for 10 mins to pellet the bacterial load. The pellet was washed three times with 50ml PBS. A final suspension of the bacteria was made in 5ml PBS.

1ml total sample was removed for the culture study (Chapter three).

2.3.4 Extraction of Genomic DNA

2.3.4.1 From Saliva samples

DNA from the saliva/PBS mixture was purified from samples according to the 'Gram-positive bacteria and yeasts' protocol from the Puregene Kit DNA extraction kit (Gentra, distributed by Flowgen, Nottingham, UK). Cells were pelleted by centrifugation at 13000 rpm (15700 x g, Eppendorf centrifuge 5415D) in 10 ml aliquots in 50 ml sterile plastic centrifuge tubes (Sarstedt). Pellets were gently resuspended in 5 ml of Cell Suspension Solution provided with the kit. 5 µl of Lytic Enzyme Solution (4,000 U/ml) was added (alternatively lysozyme at a concentration of 4mg/ml could be added); the samples were inverted 25 times and incubated at 37°C for 30 minutes (Grant incubator) to digest cell walls. After centrifugation of the samples for 1 min at 13,000 rpm (15700 x g, Eppendorf 5415D) in a bench-top centrifuge the supernatant was removed and the cells were suspended in 5 ml of Cell Lysis Solution (Puregene) and gently pipetted up and down to lyse the cells. The samples were heated at 80°C for 5 minutes to complete cell lysis. To this 15 µl Proteinase K (10µg/ml) (New England Biolabs, Hitchin, Herts, UK) was added and the sample was incubated in a water bath at 55°C for 1 h. Five µl of RNAse A Solution (4 mg/ml) (Puregene) was added to the cell lysate, the samples were mixed by inverting the tubes 25 times and then incubated at 37°C for 1 hour to remove the RNA. After cooling the samples to room temperature, 2 ml of Protein Precipitation Solution (Puregene) was added to the cell lysate and it was mixed and incubated on ice for 30-60 mins. The samples were centrifuged for 5 minutes at 14,000 rpm (15700 x g, Eppendorf 5415D) and the supernatant containing the DNA was poured into a clean 15 ml sterile centrifuge tube containing 3 ml 100% $(CH_3)_2CHOH$. The samples were inverted gently 50 times and centrifuged for 3 mins at 14,000 rpm

(15700 x g, Eppendorf 5415D). The supernatant was removed from the DNA pellet using a pipette. The pellet was then washed with 1 ml 70% C_2H_5OH (BDH Chemicals, Poole, UK) and centrifuged for 1 minute at 14,000 rpm (15700 x g, Eppendorf 5415D). The tubes were drained on 3 mm Whatman paper (Whatman, Brentford, UK) and allowed to air dry for 30 minutes. The DNA pellet was redissolved overnight in 500 µl sterile distilled water.

2.3.4.2 From Faecal Samples

The above protocol was used for the initial stage of DNA extraction from the faecal suspensions outlined in the sample processing. However, samples remained visibly dirty and the following clean-up protocol was used:

500 µl of original DNA extract was mixed with 13.5 ml DNA extraction buffer (Appendix 1) and 100 µl proteinase K (10 mg/ml) (NEB) in a 15 ml sterile plastic centrifuge tube (Sarstedt). This was incubated horizontally at 37°C with shaking (225 rpm, Sanyo Orbital Incubator) for 30 min. Following this 1.5 ml of 20% SDS was added and the samples were incubated in a 65°C water bath for 2.5 h. The reaction was then transferred to a 50 ml sterile centrifuge tube (Sarstedt) and an equal volume (15.6 ml) of chloroform-isoamyl alcohol (24:1 v/v) was added. The mixture was vortexed gently and the aqueous phase was recovered by centrifugation for 20 min at 4000 rpm (2862 x g, eppendorf 5804R). Once the contents of the tube had settled a wide-bore pipette tip (Sarstedt, cut with sterile scissors) was used to removed the upper DNA-containing phase. To this 0.6 volume (9.4 ml) $(CH_3)_2CHOH$ was added and the mixture was incubated for 1 h at room temperature. Finally the solution was centrifuged for 20 min at the highest rpm (~4000; 2862 x g, Eppendorf 5804R) and

the DNA pellet was washed with 70% C_2H_5OH (100% AnalaR BDH, diluted with sterile distilled water). This was removed with a pipette and the DNA pellet was allowed to air-dry before being resuspended in 500 µl sterile distilled water at 4°C overnight.

2.4 MIC Determination

The MIC of isolates of interest for tetracycline, erythromycin, and vancomycin was determined by plating each on iso-sensitest agar plates supplemented with 5% defibrinated horse blood containing 1µg/ml, 2µg/ml, 4µg/ml, 8µg/ml, 16µg/ml, 32µg/ml, 64µg/ml and 128µg/ml of the antibiotic. The inoculum was a single colony from an over-night streak plate which was spread on plates containing the above concentrations. Plates were read after 24 hr incubation at 37°C aerobically or 48 hr incubation at 37°C anaerobically according to the atmosphere requirement of the isolate. The highest dilution of antibiotic that inhibited visible growth was taken to be the MIC (MacGowan & Wise 2001; Andrews 2001). *E. coli* DHB10 and NCTC6571 were used as a negative controls in the tetracycline MIC point determination, and *B. subtilis* BS34A (containing *tet*(M) on Tn*916*) was used as a positive control.

2.5 PCR of *tet* Determinants

PCR reactions used plasmid DNA template prepared using the Qiagen plasmid extraction kit, described in section 2.10.1. The PCR reaction mixture (total 100 µl) included 5 µl template DNA in a final concentration of 0.1 to 1 µg, 10 µl 10 x PCR buffer (Invitrogen), 3 µl $MgCl_2$ buffer (Invitrogen), 0.3 µl DNA Taq polymerase

(Invitrogen), 300 μM of each of the deoxynucleotides dATP, dCTP, dGTP and dTTP (Invitrogen) and dH_2O.

Individual PCR reactions were performed for *tet*(M), *tet*(O), *tet*(W), *tet*(S) and *tet*(32). Primers are listed in Appendix 4 (Aminov *et al.*, 2001). PCR reactions were run on the Biometra T3000 Thermocycler (Biometra, Goettingen, Germany) using the following programme: 30 cycles of 1 min at 90°C, 1 min at 54 °C, and 1 min at 72 °C, with a final extension for 10 mins at 72 °C. The annealing step (54 °C for 1 min) was variable and altered depending on the melting temperature (Tm) of the primers used, and calculated by the following equation:

Tm = 2 °C x (A+T) + 4 °C x (G+C)

The annealing step for a particular reaction was derived as 5 °C below the Tm of the primer with the lowest Tm. The times of the elongation step (72 °C for 1 min) were dependent on the expected length of the product that is being amplified: 1 min for each 1 Kb.

2.6 16S rRNA PCR

The synthesis of all oligonucleotides was carried out by Genosys Biotechnologies (Europe) Ltd. (Pampisford, UK). Sequences of all primers used throughout this study are shown in Appendix 4.

Metagenomic/genomic/plasmid DNA was extracted and purified as described elsewhere in the materials and methods. A 1465-bp subregion of the 16S rRNA gene was amplified using primers 27F and 1492R (Appendix 4)

In a total reaction volume of 100 μl, 10 μl of 10 x buffer (Invitrogen, Paisley, UK) was mixed with 3 μl of 50 mM $MgCl_2$ (Invitrogen), 1 μl of 100 mM dNTPs mixture

(Invitrogen), 1 µl of each primer (50 pmole), 2-5 µl of template (to a final amount of 0.1 to 1 µg), X µl of sterile distilled water and 0.3 µl of Taq polymerase (Invitrogen). PCR were performed for 30 cycles of 60 s at 94°C, 60 s at 54°C, and 90 s at 72°C, with a final extension at 72°C for 5 min (Biometra T3000).

2.7 Agarose Gel Electrophoresis

DNA (metagenomic, genomic, plasmid, PCR products) were visualised by agarose (Amresco, Solo, Ohio, USA) gel electrophoresis (Sambrook *et al.*, 1989).

Gels were made up with 1 x TAE (Appendix 1) according to the volume required. A standard concentration of 1% agarose was used. The mixture was heated in a glass conical flask in a microwave (Sharp Compact) until the agarose had completed dissolved. The liquid was then cooled and 0.5 µg/ml of Ethidium Bromide was added while the mixture was still liquid. The agarose was poured into the appropriate gel tray and allowed to cool to a gel.

DNA samples were mixed with 6 x Blue/Orange Loading dye (Promega) and loaded into the gel wells. The appropriate marker, most commonly 10 kb bench top ladder (NEB) was loaded in the left hand lane. The gels were run at an appropriate voltage (70-100V) for an appropriate length of time depending on the expected size of the DNA to be visualised.

The gel was exposed to UV light in an Alpha Imager (Alpha Innotech Corporation, distributed through Flowgen) to visualise the DNA. Where appropriate a photographic record was made of the results (Alpha Imager 1220 Documentation and Analysis System).

2.8 Purification of PCR samples

Prior to sequencing, the PCR products were purified from the other components in the reaction such as excess primers, nucleotides, DNA polymerase, and salts, using the GenElute^{TM} PCR Clean-Up Kit (Sigma Aldrich). Each sample (50 µl) was mixed with 5 volumes of Binding Solution (250 µl), transferred into a GenElute PCR Clean-up miniprep column (Qiagen) and centrifuged for 1 min at 14,000 rpm (15,800 x g, Juoan A14 centrifuge). The flow-through was discarded and 500 µl of diluted Wash Solution (12 ml of Wash Solution diluted with 48 ml of 100% C_2H_5OH) was added, the sample was centrifuged for 1 min at 14,000 rpm (15,800 x g, benchtop Juoan A14 centrifuge). The flow-through was discarded and the sample was centrifuged again for 2 min. The column was transferred to a fresh eppendorf tube, 50 µl of Elution Buffer was applied to the centre of the column and each tube was incubated at room temperature for 1 min. To elute the DNA, the column was centrifuged for 1 min. The sample was run on a gel to confirm the presence of DNA before sequencing.

2.9 DNA Sequencing

2.9.1 DNA sequencing PCR

The sequencing of PCR products was carried out according to the PE Biosystems (Warrington, UK) protocol with the following modifications. In a total volume of 7 µl, 5 pmol of primers were mixed with 2 µl of 1:4 ABI BigDye Terminator Ready Reaction Mix diluted with sequence buffer (400mM Tris-HCL, pH 9.0 and 10 mM $MgCl_2$), and 1 to 4 µl of DNA sample. The samples were then run on the following program; rapid thermal ramp to 95°C, held for 10 sec, rapid thermal ramp to 50°C, held for 5 sec, rapid thermal ramp to 60°C, held for 4min. These four steps are

repeated for 99 cycles followed by a rapid thermal ramp to 4°C until removed from the PCR machine.

2.9.2 Purification of Sequence Products

15 µl of H_2O, 2 µl of 3M CH_3COONa and 50 µl -20°C 100% C_2H_5OH (100% AnalaR BDH) was added to each PCR tube. They were incubated on ice for 20 min. The samples were spun at 14000 rpm (15700 x g, Eppendorf centrifuge 5402) for 25 min at 4°C. Following that, the supernatant was removed using a pipette, and the pellet washed and centrifuged with 250 µl -20°C 70% C_2H_5OH by centrifugation for 15 min at 14000 rpm (15700 x g, Eppendorf 5402). The supernatant was removed similarly and the sample was dried at 95°C for a few seconds and resuspended in 20 µl of template suppresser reaction buffer (Applied Biosystems, Warrington, UK).

2.9.3 Sequencing of Tetracycline Resistant BAC Clone Inserts

All sequencing was performed by LARK DNA Sequence Technology Systems (Takeley, Essex).

2.10 Molecular Cloning

2.10.1 Plasmid Extraction

pUC19, BAC and pDL278 plasmids were extracted in a similar manner, both from *E. coli* hosts. Prior to extraction one colony containing the plasmid from a fresh over night plate culture was inoculated in 10 ml of broth. LB was used for pUC19 and BAC plasmids and BHI, for pDL278. Cultures containing pUC19 contained 50 µg/ml of ampicillin, strains containing BACs were supplemented with 12.5 µg/ml

chloramphenicol, and cultures containing pDL278 were supplemented with 50 μg/ml spectinomycin. Liquid cultures were incubated 37°C with shaking at 225 rpm (Stuart Scientific Orbital Shaker SO1) overnight. Bacteria were recovered by centrifugation at 6000 rpm (6800 x g, Eppendorf 5804R) for 5 min and the plasmid was extracted using the midiprep kit (Qiagen) according to the manufacturer's instructions. Plasmids were then extracted using the Qiagen plasmid mini kit.

2.10.2 Plasmid Digestion and Dephosphorylation

Plasmid digests were carried out in a total volume of 50 μl, (5μl restriction enzyme buffer, 2 μl restriction enzyme (10 units/μl), 30 μl plasmid DNA and 13 μl sterile distilled water and incubated at 37°C for 90 min.

Dephosphorylation was carried out at 37°C for 1h by adding 1 unit (U) of alkaline phosphatase at time 0 and 30 min. The mixture was inactivated with the addition of 2 μl of 0.5M EDTA (Appendix 1), incubated at 60°C for 20 min. The sample was purified using QIAquick PCR Purification Kit (Qiagen), as above, and resuspended in 30 μl of sterile distilled water.

2.10.3 Insert Preparation

For the construction of both metagenomic and single species libraries in BAC and pDL278, and for sub-cloning in pUC19, the sample DNA was digested in a final volume of 40 μl consisting of 4 μl 10 x restriction enzyme buffer, 1 μl restriction enzyme (*Hin*dIII, 10 units/μl for metagenomic libraries; *Eco*R1, 10 units/μl for pUC19) diluted 1:5 with sterile distilled water, 22 μl DNA (~50 μg/ml) and 13 μl sterile distilled water. Samples were incubated at 37°C, and depending on the vector,

were incubated for 10 mins (pUC19), for 5 mins (pDL278), or were split with 50% of the sample incubated for 1 min, and 50% of the sample incubated for 2 mins (BAC).

Samples were cleaned-up by ethanol precipitation. 4 µl of 3M CH_3COONa (Sigma Aldrich) and 100µl C_2H_5OH (100% AnalaR BDH) were added to each sample. Samples were incubated at -70°C for a minimum of 1 h. The DNA was recovered by centrifugation in a bench-top centrifuge at 14000 rpm (15700 x g, eppendorf 5415D) for 30 min. Pellets were washed with 70% C_2H_5OH (100% AnalaR BDH diluted with sterile dH_2O) and centrifuged at 14000 rpm for 15 min as detailed above. The ethanol was removed with a pipette, the pellet allowed to dry and the DNA resuspended in 30 µl sterile dH_2O.

2.10.4 Ligations

For BAC libraries the Epicentre CopyControl Ligation kit was used:

1 µl pCC1BAC (25 ng/µl) (Figure 2.1) (diluted 1:5 with sterile distilled water) (supplied as a cloning-ready vector by Epicentre), and 86 µl 50 µg/ml insert DNA were incubated on their own for an initial 10 min at 55°C to encourage linkage of cohesive ends. To this 10 µl 10 x Fast-Link Ligation Buffer, 1 µl 10mM ATP, and 2 µl Fast-Link DNA Ligase were added and incubated at 16°C over night. The DNA Ligase was denatured by heating to 70°C for 15 mins.

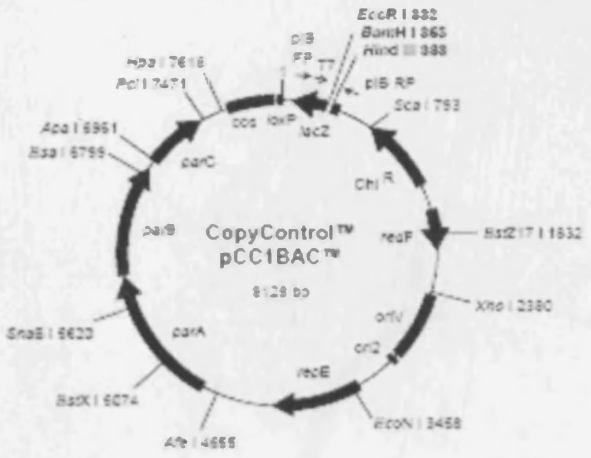


Figure 2.1: pCC1BAC vector used to create single species and metagenomic libraries in *E. coli*. Primers for sequencing are indicated with small black arrows: p1B FP-1 (forward primer for insert sequencing), p1B RP-1 (reverse primer) (from the Epicentre CopyControl BAC Cloning kit manual).

For pDL278 (Figure 2.2) and pUC19 cloning and sub-cloning, the insert and the vector (dephosphorylated) were mixed together in a total volume of 100 µl: including 2µl T4 DNA ligase (3 units/µl) and 10 µl of 10 x T4 DNA ligase buffer. The insert:vector concentration ratio was kept at approximately 3:1. The sample was incubated overnight at 16°C and then at 60°C for 20 min to inactivate the reaction.

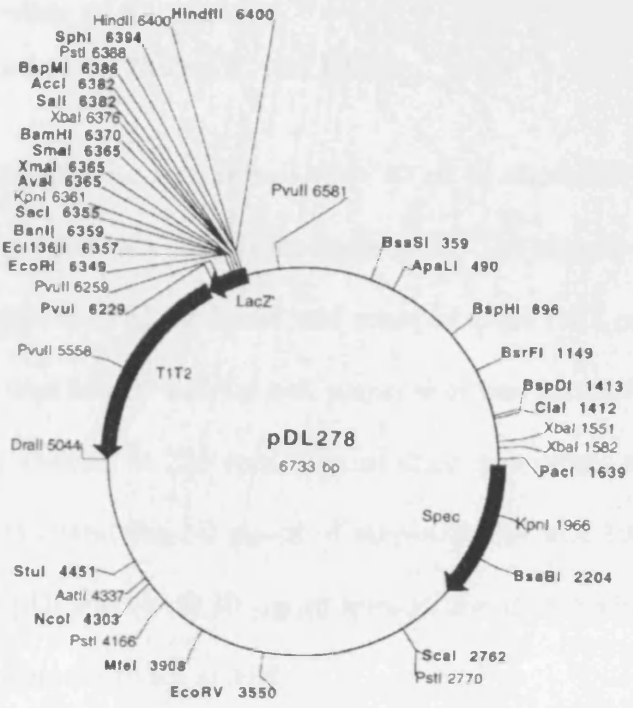


Figure 2.2: pDL278 vector used for cloning in *S. mutans* (Dunny *et al.*, 1991). Black arrows indicate genes and their direction of transcription (spec; spectinomycin resistance gene); thin black lines indicate restriction sites.

2.10.5 Desalting

All ligation reactions, regardless of method used were desalted on agarose cones (0.9 g glucose, 0.5 g agarose, 50 ml water heated to dissolve, dispensed into eppendorfs) for 1 h, on ice prior to transformation.

2.11 Transformation

2.11.1 Electroporation of *E. coli*

2.11.1.1 pUC19 and pDL278 into *E. coli* DH5α

5 µl of the ligation mixture was mixed with 50 µl of chemically competent cells (DH5α sub-cloning efficiency cells from Invitrogen). The sample was incubated on ice for 30 min, heated at 37°C for 1 min, and returned to ice for 2 min. To this 950 µl SOC (Invitrogen) was added, and the cell suspension was incubated at 37°C for 90 min with constant shaking at 225 rpm. The mixture was plated onto selective LB plates (Appendix 1) containing 50 µg/ml of ampicillin for pUC19 and 50 µg/ml of spectinomycin for pDL278 (with 10 µg/ml tetracycline if screening for resistance), and they were incubated for 18 h at 37°C.

2.11.1.2 BAC into *E. coli* EPI300-T1^R

Cells were transformed according to the BAC CopyControl kit (Epicentre) protocol: 5 µl of desalted ligation product were mixed in an eppendorf with 50 µl just-thawed competent EPI300 *E. coli*. The mixture was transferred to a pre-chilled electroporation cuvette 0.1 cm (Bio-Rad, Hemel Hempsted, UK). The mixture was immediately electroporated at 1.7 kV, 25uF, 200Ω (BioRad Gene Pulser II electroporater). Following eletroporation, 950 ml fresh SOC (Invitrogen) was added to the cells and they were allowed to recover for 1 h at 37°C with shaking at 225 rpm (Sanyo Orbital Shaker), before plating on selective medium (LB + 12.5 µg/ml chloramphenicol, 40 µg/ml X-Gal, 0.4 mM IPTG).

2.11.2 Transformation of *S. mutans* with Competence Stimulating Peptide (CSP)

An overnight culture of a single fresh *S. mutans* colony was grown in 10 ml BHI broth containing 5% heat inactivated horse serum without antibiotics in 5% CO_2 (LEEC cabinet, with CO2 from BOC gases, Guildford, UK).

The culture was diluted 1:20 into 10 ml of fresh pre-warmed BHI broth and incubated in the CO_2 incubator until the OD_{600} = 0.2. Five µg Competence Stimulating Peptide (CSP), MKKTLSLKNDFKEIKTDLEIIIGG^SGSLSTFFRLFNRSFTQALGK (Sigma Genosys) was added to the 10 ml culture, and 1 ml aliquots of culture were added to the DNA to be transformed (5 µl high-concentration vector). Aliquots were returned to the CO_2 cabinet and incubation continued for 90 mins.

Cells were concentrated 5 times by centrifugation (6000 rpm / 6800 x g, Eppendorf 5804R).

2.12 Determining the Coverage of a BAC Library

The following equation (taken from the Epicentre BAC CopyControl Cloning Kit Manual) was used to establish the 'coverage' of each library i.e. the likelihood of any one sequence occurring in the library.

$$N = \ln(1-P) / \ln(1-[I/GS])$$

Where:

P = Desired probability (expressed as a fraction)

I = Average insert size of the library

N = Number of clones required

GS = Genome size (average microbial genome size was taken to be 4.5 Mb (based on sequences in the database at the time of calculation) multiplied by the estimated number of organisms in the microbiota

2.13 Induction of BAC Clones to High-Copy Number

1 ml aliquots of LB + 12.5 μg/ml chloramphenicol were inoculated with single BAC colonies. Cultures were incubated at 37°C overnight without shaking. Following this incubation, each tube was vortexed and 800 μl was removed and discarded. 800 μl fresh LB + 12.5μg/ml chloramphenicol was added to the remaining culture and the tubes were incubated for 30 min at 37°C with shaking at 250 rpm. Following this 1 μl 1000 x CopyControl Induction Solution (Epicentre) was added to each tube. The cultures were incubated horizontally (to ensure maximum aeration) for 2 h at 37 °C with shaking at 250 rpm.

2.14 Screening of Clones

2.14.1 Screening on Selective Media

BAC and pDL278 containing clones were plated directly onto selective agar. BAC clones were plated onto LB agar containing 12.5 μg/ml chloramphenicol, 10 μg/ml tetracycline. pDL278 clones (in *S. mutans*) were screened on 800 μg/ml spectinomycin and 8 μg/ml tetracycline.

2.14.2 Macroarray Analysis of Clones

The Macroarray developed by Patterson *et al.*, (ahead of publication) was used for all samples. For the antibiotic resistant isolates and clones of interest, the samples were spotted on the membrane, as detailed below. For the array analysis of genes present in the crude DNA from each environment the probes were spotted on the membranes, and the sample DNA labelled and used as the 'probe'. The arrangement of probes on the membrane is shown in Figure 2.3.

16S rDNA		*tet*(Q)	*tet*(A)	*tet*(H)	*tet*(34)		*erm*(A)			16S rDNA
		tet(S)	*tet*(B)	*tet*(J)		Tn *916* integrase	*erm*(B)	*erm*(G)		
	tet(M)	*tet*(T)	*tet*(C)	*tet* A(P)		Tn*B* *1230* orf1	*erm*(C)	*erm*(Q)	Tox A	
	tet(O)	*tet*(W)	*tet*(D)	*tet*(Y)		Tn *1549* integrase	*erm*(D)	*erm*(V)	Tox B	
	*tet*B(P)	*tet*(32)	*tet*(E)	*tet*(Z)		Tn *4451* Recombinase	*erm*(E)	*erm*(X)	Entero -tox	
		tet(36)	*tet*(G)	*tet*(30)	*tet*(X)	Tn *5397* resolvase	*erm*(F)			
16S rDNA										16S rDNA

Figure 2.3: Layout of probes on the macroarray when probing with metagenomic DNA

2.14.2.1 Printing the Array

400µl of each isolate/clone glycerol stock was spun down in a bench-top centrifuge at top speed for 2 min (~14000 rpm, 15700 x g, Eppendorf 5402). Samples were resuspended in 60µl sterile dH_2O. The cell suspensions were lysed and the DNA denatured by boiling in a water bath for 10 min. Samples were placed in a pre-assigned grid in a round-bottomed microtitre plate.

Samples were printed onto Biodyne B pre-cut modified, 0.45µm nylon membranes (Pierce, Cheshire, UK) using the Biorobotics Microgrid II System. They were dried and cross-linked using the Bio-Rad GS Gene Linker (Bio-Rad) for 20 sec.

2.14.2 Labelling the Probe

PCR products of genes of interest were used as probes. 500ng of probe was suspended in 11.5µl of sterile distilled water. Probes were boiled in a water bath for 10 min to denature the DNA. They were placed on ice for 10 mins. 1µl of each of dATP, dGTP, and dTTP; 2µl of reaction mix; 1µl klenow enzyme and 2.5µl 32P dCTP were added to the probes. The mixtures were incubated on a heat block for 30 min at 37°C.

In a separate sterile corex tube 10µl herring sperm, 970µl dH20, and 20µl 32P mix were mixed and placed in a boiling water bath for 10 mins. This was poured on the prehybridised membrane.

2.14.3 Prehybridisation and Hybridisation

1g dextran sulphate, 9ml dH_2O and 1ml 10% SDS were mixed in a sterile corex tube and warmed to 65°C. 0.58g NaCl was added, and kept at 65°C.

The nylon membranes were dampened with water and placed in hybridisation tubes. The above mixture was added to these tubes and they were placed in the hybridisation oven at 65°C with turning for 30 mins before the radioactive probe mixture was added.

Upon addition of the probe, the membranes were returned to the oven and hybridised overnight.

2.14.4 Washes

The probe mixture was disposed of and the membranes washed twice with 100ml 2 x SSC for 5 mins; twice with 200ml 2 x SSC + 1% SDS for 30 mins; twice with 200ml 1 x SSC + 0.5% SDS for 30 mins; and finally twice with 100ml 1 x SSC for 5 mins. Membranes were then placed between Saran wrap and secured in place in a Fujifilm BAS 2340 cassette. A Fuji Imaging plate was placed on top of the membrane and the cassette closed while the film developed. All washes were conducted at room temperature with gentle agitation.

2.14.5 Signal Generation and Detection

The films were exposed for 24h, and a second film was used for a second exposure from 24h – 3 weeks. The films were removed from the cassettes and immediately placed in a Fujifilm FLA-3000 scanner.

2.14.3 Colony Blotting – ECL Direct Nucleic Acid Labelling and Detection System

2.14.3.1 Blotting Colonies onto the Membrane

The BAC/pUC19/pDL278 libraries of isolates of interest were screened using the colony blot protocol of the Amersham Biosciences ECL direct nucleic acid labelling system (Amersham Biosciences, Buckinghamshire, UK). Libraries were plated out to give ~200-300 colonies per plate on the appropriate medium.

The Hybond-N+ membrane (Pierce) was cut into discs and laid on the agar plates for 30 s until the disc was completely wet (orientation was marked with a pencil). The discs were placed, colony side up for 5 min on two sheets of Whatman 3mm paper saturated with 0.5M NaOH to fix the DNA.

The discs were rinsed twice by gentle agitation in a dish containing 400ml 5 x SSC for 1 min. They were then allowed to dry by placing them DNA side up on 3mm Whatmann paper.

2.14.3.2 Labelling of DNA Probes

The DNA to be labelled was diluted to a concentration of 10 ng/µl using sterile distilled water. 10 µl of each probe was denatured by boiling in a water bath for 5 min followed immediately by 5 min incubation on ice. The contents of the tube were

then briefly centrifuged to collect them in the bottom of the tube. An equivalent volume (10 µl) of DNA labeling reagent (Amersham) was added to the cooled DNA and mixed gently but thoroughly. 10µl gluteraldehyde solution was added and mixed thoroughly. The contents of the tube were again spun. The tube was incubated for 10 min at 37°C and, if not used immediately was held on ice for 10-15 min.

2.14.3.3 Hybridisation and Stringency Washes

The hybridisation buffer was prepared as follows: solid analytical grade NaCl was added to the required volume of hybridisation buffer (50 ml) to a final concentration of 0.5 M. Blocking reagent was then added to a final concentration of 5% (w/v) and immediately mixed until the blocking reagent was present as a free suspension. Mixing continued with a magnetic stirrer for 1 h at room temperature and was followed by incubation at 42 °C for 0.5-1.0 h with occasional mixing. If the buffer was not being used immediately it was stored at –20 °C for 3 months.

2.14.3.4 Prehybridisation and Hybridisation

The blots (damped with 5 x SSC in order to arranged them in an appropriate position) were placed in a hybridisation tube. The hybridisation buffer, at 42 °C, was added (0.0625 – 0.125 ml/cm^2), and the blot was pre-hybridised for 2 h at 42 °C in a Biometra (Luton, UK) OV3 rotisserie oven.

The labelled probe was added to the buffer, taking care not to place it directly on the membrane. In some cases, a small volume of the prehybridisation buffer was removed for mixing with the probe before its reintroduction. Hybridisation was carried out for 4 h in the rotisserie oven at 42 °C.

2.14.3.5 Washes

The appropriate volume of primary wash buffer containing urea (Appendix 1) was warmed to 42 °C. The hybridisation buffer in the tube was discarded and 50-100 ml of 5 x SSC added. The tube was replaced in the rotisserie oven and incubated for 20 min at 42 °C. The primary wash was repeated. The blots were then removed from the hybridisation tube and washed twice in an appropriate container in an excess of secondary wash buffer (2 x SSC), with gentle agitation on a rotary shaker for 5 min.

2.14.3.6 Signal Generation and Detection

An equal volume of ECL reagent 1 and ECL reagent 2 (Amersham) were mixed in a 20 ml universal tube. The amount mixed was sufficient to cover the blot (0.125 ml/cm2). The excess secondary wash buffer was drained from the blot and the blot was placed DNA side up onto a clean piece of Saran Wrap. The detection reagents were poured onto the blot immediately and incubated at room temperature for 1 min with gentle agitation. The excess detection reagents were drained from the blot and it was wrapped in Saran Wrap. Air pockets were smoothed out and the blot was placed in a film cassette DNA side up. Autoradiography film (Hyperfilm, Amersham) was placed over the blot. The film cassette was closed and the film exposed for 1-3 h. The film was removed under safe light conditions and developed.

2.15 Bioinformatics

DNA sequences were edited using the Chromas 1.45 software, and analysed with the DNA-MAN version 5.2.2 program (Lynnon Biosoft). Similarity analysis was carried out with the Advance Blast program of GenBank (National Centre for Biotechnology

Information, National Institutes of Health, Washington, DC), open reading frames (orfs) were identified with ORF finder (www.bioinformatics.vg/sms/orf_find.html), and appropriate ribosome binding sites were identified by eye. Alignments were performed using the CLUSTAL W program service at the European Bioinformatics Institutes (http://www.ebi.ac.uk/). G+C content was analysed using the GeeCee program of http://www.bioinformatics.net.

Macroarray images were analysed using the AIDA Metrix alignment software, and hybridisation intensities were determined to be a percentage of the hybridisation signal, detected using this software, of the 16S rRNA control.

2.16 Statistical Analysis

The difference between average plate counts on each antibiotic, for each country were analysed using the student's *t*-test in the SPSS statistical package (SPSS Inc. (2000) 10.0 Syntax reference guide, Chicago, IL).

CHAPTER THREE

Prevalence of tetracycline, erythromycin and vancomycin resistance determinants in the Gram positive aerobic and facultative anaerobic cultivable portion of the human oral and faecal microbiota from six European countries.

3.1 Introduction.

It is widely documented that the human GI tract is a reservoir of resistance determinants (Ready *et al.*, 2003; Roberts, 1998a, 1998b; Millar *et al.*, 2001) that have the ability to be transferred to and from transient organisms and subsequently to bacteria in the environment including soil and marine bacteria due to contamination by effluent (Eaton & Gasson, 2001). They are then able to spread throughout a population due to their presence on mobile genetic elements (Cooper *et al.*, 1996; Shoemaker *et al.*, 1985; Roberts, 1998a). Additionally the presence in both oral and intestinal environments of naturally competent bacteria (e.g. *Streptococcus* spp. and *Neisseria* spp.) (Stone & Kwaik, 1999; Solomon & Grossmann, 1996) will have an impact on the dissemination of resistance genes throughout these species in these environments (Wang *et al.*, 2002).

Tetracycline has long been used in the treatment of a wide range of human infections, including atypical pneumonia, cholera, periodontal infection, acne and many other genital, local and systemic infections (Roberts, 1998) It is also commonly used against oral infections and as a prophylactic agent in dentistry (reviewed in Chopra & Roberts, 2001; Fine *et al.*, 1998). Erythromycin is a macrolide antibiotic used in the treatment of infections of lungs and throat and some STIs (Sexually Transmitted Infections) (reviewed in Goldman and Scalglione, 2004). Vancomycin is a bacteriocidal antibiotic used clinically in the treatment of multi-drug-resistant infections caused by Gram positive bacteria (Boneca & Chiosis, 2003). All can be administered orally, thus exerting a selective pressure on the bacteria of the GI tract (Edlund & Nord, 2000; Ready *et al.*, 2002).

The purpose of this part of the study was to determine the levels of resistance to the above antibiotics, and establish the genetic basis of tetracycline and erythromycin resistance in Gram positive isolates by macroarray analysis. The results obtained are important for establishing any differences between the basis for resistance in the cultivable portion of the microbiota compared to the entire microbiota. Additionally the data may indicate trends in the presence of different genes or levels of resistance found in different environments (i.e. oral and faecal).

3.2 Materials and methods.

3.2.1 Culture of Aerobic and Facultative Anaerobic Bacteria from Samples

A 10-fold serial dilution of 1ml total sample was prepared in Luria Bertani broth (Appendix 1) and spread onto both antibiotic-containing and antibiotic-free Isosensitest (5 % agar to prevent swarming of *Proteus* spp. (Jeffries & Rogers 1968)) agar plates to determine the total number of cultivable bacteria in the specimen in duplicate (petri dishes from Sarstedt). The concentrations of antibiotics used were 2μg/ml (tetracycline) 1μg/ml (erythromycin) and 8μg/ml (vancomycin) (Appendix 5). Growth above these points is defined as resistant by the British Society for Antimicrobial Chemotherapy (BSAC) (MacGowan & Wise 2001; Andrews 2001). Iso-sensitest agar supplemented with 5% defibrinated horse blood (E&O Laboratories, Bonnybridge, UK) was used for microaerophilic incubation (air + 5% CO_2); Wilkins-Charlgren agar supplemented with 5% defibrinated horse blood was used for facultative anaerobic incubation.

3.2.2 Enumeration and storage - Identification of Antibiotic Resistance Bacteria

Identification of isolates was initially done by Gram staining (Pro-lab diagnostics reagents). Gram positive isolates were identified to the species level, using partial 16S ribosomal RNA (rRNA) gene sequencing (Lane *et al.*, 1985) outlined in Chapter two.

Gram positive aerobic isolates were cultured in 10ml iso-sensitest broth containing the relevant antibiotic at 37°C, overnight with shaking at 250 rpm (Stuart Scientific Orbital Shaker SO1). Gram positive facultative anaerobic isolates were cultured in

WC broth plus the relevant antibiotic at 37°C for three days. 900µl of culture was added to a 1.5ml cryogenic tube (Nalgene). To each 100µl sterile glycerol was added. Tubes were vortexed for 30 secs and frozen immediately at –70°C.

3.3 Results

3.3.1 Gram Positive Resistant Isolates.

Following Gram staining to select for Gram positive bacteria 123 isolates were collected and identified to genus level (Appendix 6). Seventy nine were isolated from the oral samples, 44 from the faecal samples. Of these 123 isolates, 107 were tetracycline resistant. Sixty nine of the 107 (63.9%) were oral isolates and 39 (36.1%) were faecal isolates). In addition, 105 of the 123 Gram positive isolates were erythromycin resistant, and included 70 (66.7%) oral isolates and 35 (33.3%) faecal isolates. No vancomycin resistant Gram positives were isolated from the samples. Eighty four isolates (66.7 % of the total) were resistant to both tetracycline and erythromycin.

3.3.2 Oral Isolates

In the oral samples, the most commonly isolated aerobic / facultative anaerobic genus from the tetracycline and/or erythromycin resistant portion of the microflora was the streptococci (74.6% of isolates), followed by the staphylococci (7.6%). *Bacillus* spp. and *Actinomyces* spp. which both accounted for 5.1 % of resistance isolates. Other species accounted for 7.6 % of total resistant isolates and included *Acidobacterium* sp. (2 isolates), *Lactobacillus* sp. (2 isolates), *Rothia* sp. (1 isolate) and *Enterococcus* sp. (1 isolate) (Table 3.1).

3.3.2.1 Tetracycline resistance in the Aerobic / Facultative Anaerobic Cultivable Oral Flora

The total percentage of tetracycline resistance from each sample are shown in Figure 3.1. In the oral cavity levels tetracycline resistance varies between countries from 8.55 % of the total aerobic / facultative anaerobic cultivables in the Scottish samples, to 30.6 % in the Finnish samples. However, using the student's t-test (P = >0.01), there was no significant difference between the levels of resistance in different countries.

of

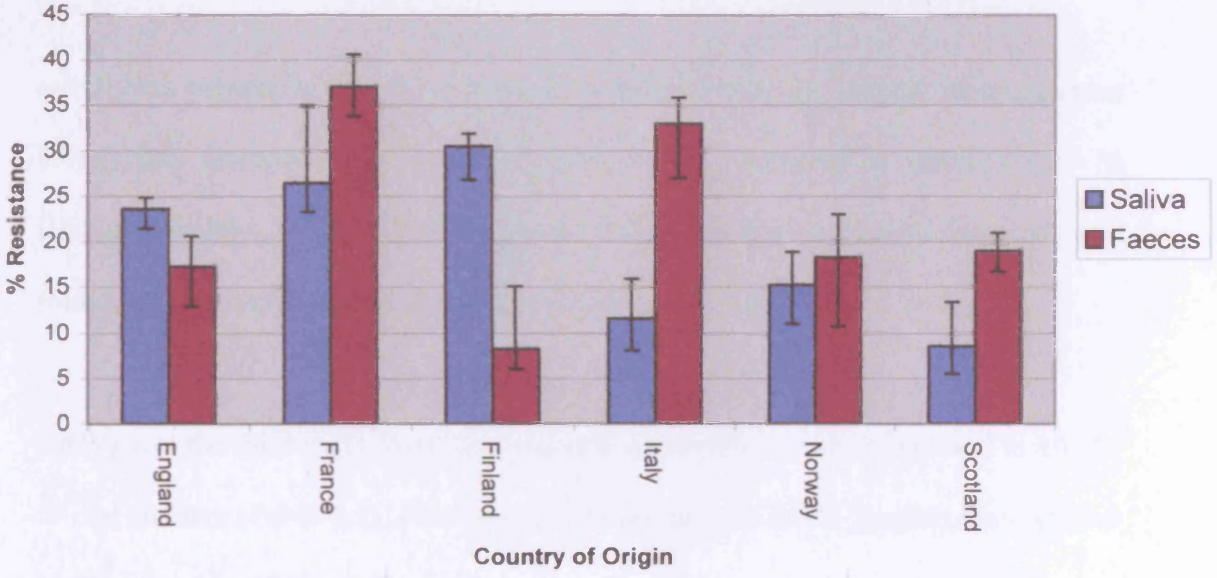


Figure 3.1: Tetracycline resistance profiles of the cultivable aerobic / facultative anaerobic oral and faecal microflora of six European countries.

3.3.2.1.1 Genetic Basis of Tetracycline Resistance

Genomic DNA from each isolate was blotted onto the nylon membranes of the array and fixed as described in section 2.12.2.1. The membranes were then probed with the *tet* gene-specific probes to determine the genetic basis of resistance in each isolate.

tet(M) was the most common *tet* gene found. It was present in 76.8% of the oral tetracycline resistant isolates (Table 3.1). Levels of the gene in individual countries ranged from 28.6% in the Norwegian sample to 82.4% in the Scottish sample (Table 3.2).

tet(O) was present in 66.7% of the oral isolates. It was the second most common tetracycline resistance gene isolated (Table 3.1). It occurred in between 41.7 % (Italian sample) - 100 % (English sample) of tetracycline resistant isolates and was found in every sample set from the oral cavity (Table 3.2).

tet(W) was the third most frequently isolated tetracycline gene. It occurred in 10.1% of oral isolates (Table 3.1). However, it was not present in the English, Finnish and Scottish samples (Table 3.2).

Probes for other RPP genes (*tet*B(P), *tet*(S), *tet*(T), *tet*(W), *tet*(32) and *tet*(36) but not *tet* and *otr*(A)) genes which were not probed for on the array) were combined in one hybridisation and were found to be present in 34.7 % of Gram positive aerobic / facultative anaerobic isolates. 18.8 % harboured an efflux gene (*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*A(P), *tet*(Y), *tet*(Z) and *tet*(30) (combined in

one hybridisation) but not *tet*(K), *tet*(L), *tet*(V), *tet*(31), *tet*(33), *tet*(35), *tet*(38), *tet*(39), *tcr*3, *otr*(B) and *otr*(C) which are not on the array).

	tet(M)	*tet*(O)	*tet*(Q)	*tet*(W)	*tet*(32)	RPP	Tetracycline Efflux	*erm*(B)	*erm*(E)	*erm*(F)	*erm*(V)	Other *erm* genes
Streptococcus spp. (59)	44 (63.8%)	37 (53.6%)		1 (1.4%)	2 (2.9%)	16 (23.2%)	12 (17.4%)	43 (61.4%)	1 (1.4%)	4 (5.7%)		4 (5.7%)
Staphylococcus spp. (6)	1 (1.4%)	2 (2.9%)		1 (1.4%)		1 (1.4%)		2 (2.9%)	1 (1.4%)	1 (1.4%)		3 (4.3%)
Bacillus spp. (4)	3 (4.3%)	3 (4.3%)		1 (1.4%)		2 (2.9%)		1 (1.4%)				1 (1.4%)
Actinomyces spp. (4)	3 (4.3%)	3 (4.3%)		1 (1.4%)		2 (2.9%)		2 (2.9%)	1 (1.4%)		1 (1.4%)	1 (1.4%)
Others (6)	2 (2.9%)	1 (1.4%)	1 (1.4%)	3 (4.3%)			1 (1.4%)	2 (2.9%)	1 (1.4%)			2 (2.9%)
Total (79)	53 (76.8%)	46 (66.7%)	1 (1.4%)	7 (10.1%)	2 (2. 9%)	21 (30.4%)	13 (18.8%)	50 (71.4%)	4 (5.7%)	5 (7.1%)	1 (1.4%)	11 (15.7%)

Table 3.1: Tetracycline and erythromycin resistance determinants isolated from the human oral cavity.
Percentages given are the percentage of total tetracycline, or erythromycin resistant isolates represented by the particular determinant.

	England	France	Finland	Italy	Norway	Scotland
tet(M)	71.4 %	54.5 %	75.0 %	58.3 %	28.6 %	82.4 %
tet(O)	100 %	62.5 %	63.6 %	41.7 %	57.1 %	75.0 %
tet(W)	0 %	6.3 %	0 %	33.4 %	28.6 %	0 %
erm(B)	75.0 %	72.7 %	100 %	42.9 %	100 %	62.5 %
erm(F)	0 %	5.5 %	11.1 %	0 %	12.5 %	0 %
erm(E)	25.0 %	0 %	0 %	14.3 %	0 %	6.3 %

Table 3.2: The proportion of tetracycline and erythromycin resistant isolates from the aerobic, Gram positive cultivable oral flora of six European countries harbouring specific determinants.

3.3.2.1.2 Isolate FStet12

One streptococcus isolate (FStet12) from the Finish oral sample hybridised to the *tet*(32) probe, but not the *tet*(O) probe in addition to the probe for 'other efflux' genes. This isolate had an MIC of 10 μg/ml in air. A BAC library was made from DNA from this isolate (Table 3.5) in order to obtain the full gene, which would then be sequenced to determine whether or not it was a hybrid gene, and the nature of its genetic support. ~36 Mb of insert (average insert size 30 Kb) was screened on selective agar (8 μg/ml tetracycline). No tetracycline resistant colonies were isolated. Four large agar plates with ~400 clones on each were subject to colony hybridisation using a PCR derived *tet*(32) probe. Therefore ~48 Mb of insert (equivalent to 23.6 streptococcal genomes, based on the genome of *S. mutans* UA159 (2.03 Mb)) was screened. No clones harbouring *tet*(32) were found.

A small insert library was made in pDL278 with an average insert size of 8 Kb. This was transformed into *S. mutans* and ~32.5 Mb of insert (16.0 streptococcal genomes) was screened on selective agar (4 μg/ml tetracycline). No tetracycline resistant colonies were isolated.

3.3.2.2 Erythromycin resistance in the Aerobic / Facultative Anaerobic Cultivable Oral Flora

Total percentages of erythromycin resistance in the oral cavity varies from 14.4 % of the total cultivable aerobic / facultative anaerobic bacteria in the English sample to 32.1 % in the Finnish sample (Figure 3.2). However, these Figures include the intrinsically resistant Gram negative bacteria as no attempt was made to exclude these from the viable counts on selective media.

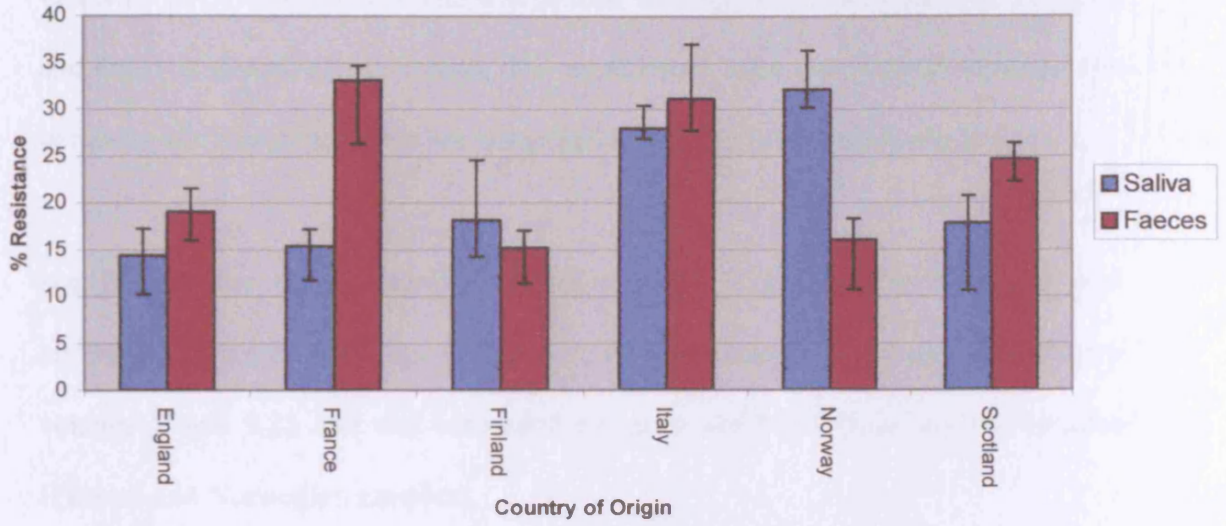


Figure 3.2: Erythromycin resistance profiles of the aerobic / facultative anaerobic oral and faecal cultivable flora of six European countries.

50 isolates from each sample were Gram stained and the percentage Gram positives was used to calculate levels of resistance among the Gram positive population (Figure 3.3).

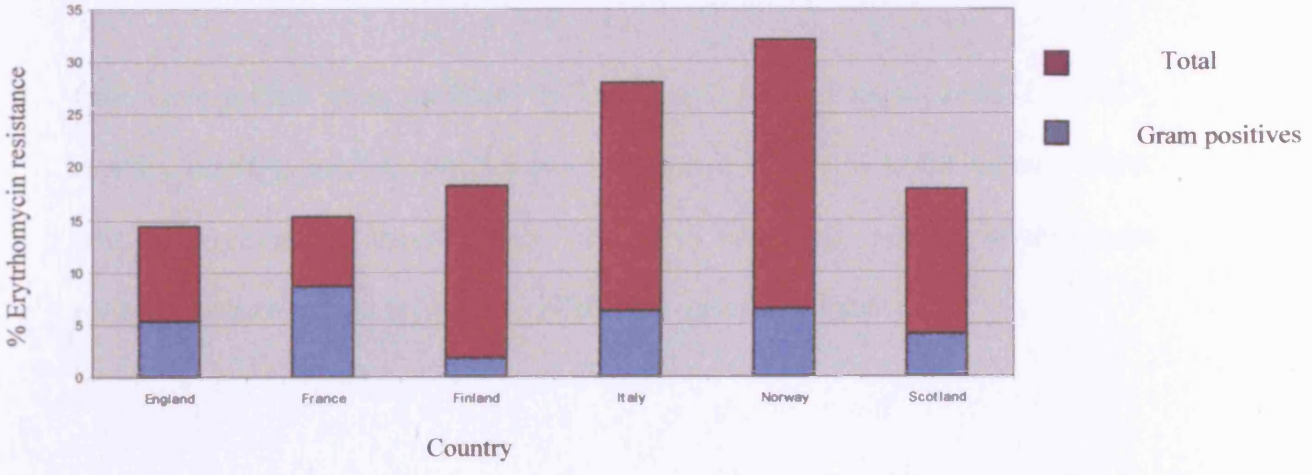


Figure 3.3: Erythromycin resistance levels in the total cultivable aerobic / facultative anaerobic flora, and the relative contribution of Gram positives

3.3.2.2.1 Genetic Basis of Erythromycin Resistance

Genomic DNA from each isolate was blotted onto the nylon membranes of the array and fixed as described previosuly. The membranes were then probed with the *erm* gene-specific probes to determine the genetic basis of resistance in each isolate.

erm(B) was the most common *erm* determinant. It occurred in 71.4% of oral erythromycin resistant isolates (Table 3.1). It was present in the samples from every country (Table 3.2), and was harboured by up to 100 % of Gram positive isolates (Finnish and Norwegian samples).

erm(F) was the second most frequently found *erm* gene. It was present in 7.1% of oral isolates (Table 3.1). It was found in one isolate from each of the Finnish, French and Italian oral samples (Table 3.2).

erm(E) was found in 5.7% of the oral isolates making it the third most commonly isolated *erm* gene (Table 3.1). However, it was absent in the French, Finnish and Norwegian oral isolates (Table 3.2).

Other *erm* probes were combined in one hybridisation (*erm*(A), *erm*(C), *erm*(D), *erm*(G), *erm*(Q), *erm*(V), *erm*(X)) and were found in 15.7 % of the Gram positive oral erythromycin resistant isolates. 12.9 % of oral Gram positive erythromycin resistant isolates did not contain any of the *erm* genes listed above.

3.3.3 Faecal Isolates

From the faecal samples the most commonly isolated resistant aerobic / facultative anaerobic genus was the enterococci, accounting for 47.7 % of the faecal isolates (Table 3.3) followed by the streptococci (27.3 % of faecal resistant isolates), and the *Staphylococcus* spp. (18.2 %). Other genera made up the remaining 15.9 % and included *Lactobacillus* spp. (2 isolates), *Eggerthella* sp. (2 isolates), *Pediococcus* sp. (1 isolate), *Citrobacter* sp. (1 isolate), and *Comonomas* sp. (1 isolate).

	tet(M)	*tet*(O)	*tet*(Q)	*tet*(W)	*tet*(32)	RPP	Efflux	*erm*(B)	*erm*(E)	*erm*(F)	*erm*(V)	Other *erm* genes
Enterococcus spp. (21)	13 (33.3%)	12 (30.8%)	1 (2.6%)			8 (20.5%)	1 (2.6%)	14 (40.0%)				1 (2.6%)
Streptococcus spp. (12)	9 (23.1%)	9 (23.1%)					1 (2.6%)	2 (5.7%)		1 (2.6%)	1 (2.6%)	
Staphylococcus spp. (8)	4 (10.3%)	3 (7.7%)	1 (2.6%)			3 (7.7%)		3 (8.6%)				4 (11.4%)
Lactobacillus spp. (2)			1 (2.6%)									
Others (5)		1 (2.6%)	1 (2.6%)	1 (2.6%)					2 (5.7%)	1 (2.6%)		
Total (44)	26 (66.7%)	25 (64.1%)	4 (10.3%)	1 (2.6%)		11 (28.2%)	2 (5.1%)	19 (54.3%)	2 (5.7%)	2 (5.7%)	1 (2.6%)	5 (14.3%)

Table 3.3: Tetracycline and erythromycin resistance determinants isolated from the human faecal flora.
Percentages given are the percentage each group contributes to total tetracycline/erythromycin resistance for this environment.

	England	France	Finland	Italy	Norway	Scotland
tet(M)	66.7 %	100 %	33.4 %	50.0 %	50.0 %	63.6 %
tet(O)	63.6 %	33.4 %	66.7 %	33.4 %	0 %	83.3 %
tet(W)	9.1 %	0 %	0 %	0 %	0 %	0 %
erm(B)	85.7 %	0 %	33.3 %	14.3 %	0 %	0 %
erm(F)	0 %	0 %	0 %	12.5 %	50.0 %	0 %
erm(E)	0 %	0 %	0 %	25.0 %	0 %	0 %

Table 3.4: The proportion of tetracycline and erythromycin resistant isolates from the cultivable faecal flora of six European countries harbouring specific determinants.

3.3.3.1 Tetracycline Resistance in the Aerobic / Facultative Anaerobic Cultivable Faecal Flora

The total proportion of viable tetracycline resistant bacteria isolated from each sample is shown in Figure 3.1. Levels of resistance varied, ranging from 8.24 % (Scotland) to 37.1 % (France). However, no significant difference between countries was found using the student's t-test (P = >0.01).

3.3.3.1.1 Genetic Basis of Tetracycline Resistance

Genomic DNA from each isolate was blotted onto the nylon membranes of the array and the genetic basis of tetracycline resistance was determined as described for the oral samples.

66.7% of faecal tetracycline resistant isolates harboured *tet*(M) making it the most common tetracycline resistance gene isolated (Table 3.3). It was found in the samples from all countries at levels ranging from 33.4 % (Finland) - 100 % (France) of Gram positive tetracycline resistant isolates (Table 3.4).

tet(O) was the second most common faecal *tet* gene, present in 64.1% of faecal isolates (Table 3.3). It was found in all samples except that from Norway (Table 3.4)

tet(Q) was the third most commonly isolated tetracycline resistance determinant, occurring in 10.3 % of tetracycline resistant isolates (Table 3.3).
tet(W) was absent in all faecal samples except the set from England in which 9.1% of tetracycline resistant isolates harboured it (Table 3.4).

Other RPP genes (*tet*B(P), *tet*(S), *tet*(T), *tet*(W), *tet*(32) and *tet*(36) but not *tet* and *otr*(A)) were found in 28.2 % of tetracycline resistance faecal isolates, and other efflux genes (*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*A(P), *tet*(Y), *tet*(Z) and *tet*(30) but not *tet*(K), *tet*(L), *tet*(V), *tet*(31), *tet*(33), *tet*(35), *tet*(38), *tet*(39), *tcr*3, *otr*(B) and *otr*(C)) in 5.1 % of isolates (Table 3.3).

3.3.3.2 Erythromycin Resistance in the Aerobic / Facultative Anerobic Cultivable Faecal Flora

Total percentages of erythromycin resistance in the cultivable aerobic / facultative anaerobic faecal flora varies from 15.2 % (Finland) to 33.1 % (France) (Figure 3.2). However, these Figures include the intrinsically resistant Gram negative bacteria as no attempt was made to exclude these from the viable counts on selective media.

50 isolates from each sample were Gram stained and the percentage Gram positives was used to calculate levels of resistance among the Gram positive population (Figure 3.4).

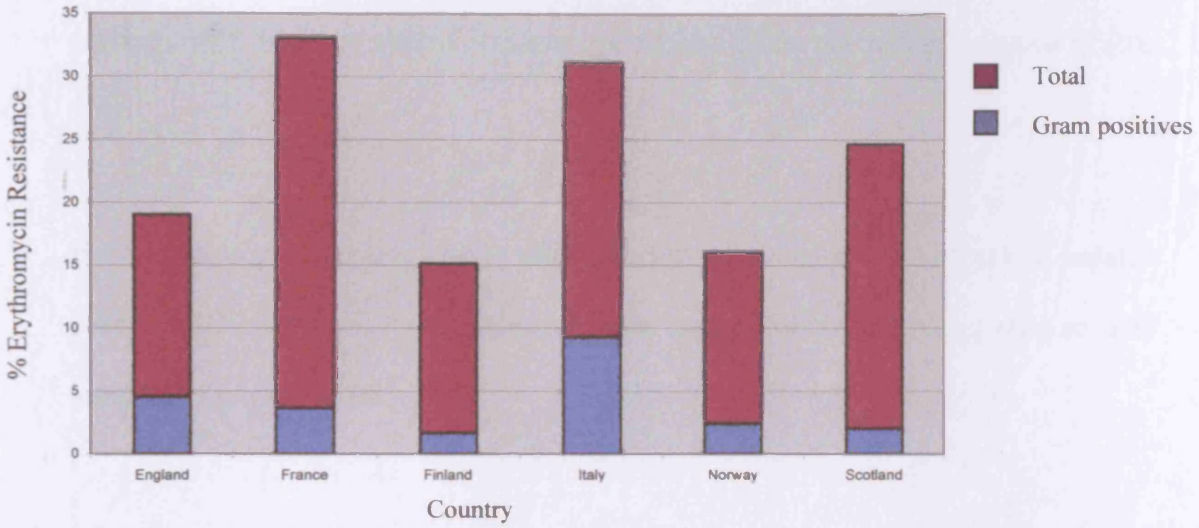


Figure 3.4: Erythromycin resistance levels in the total cultivable aerobic / facultative anaerobic faecal flora, and the relative contribution of Gram positives.

3.3.3.2.1 Genetic Basis of Erythromycin Resistance

The genetic basis of erythromycin resistance was determined by Macroarray hybridisations as described for the oral samples (section 3.3.2.2.1).

erm(B) was the most common faecal *erm* gene. It occurred in 54.3% of the total faecal erythromycin resistant isolates (Table 3.3). It was not found at all in the Scottish, French and Norwegian faecal samples, but was present in the other faecal samples (Table 3.4).

erm(F) was the second most frequently found *erm* gene. It was present in 5.7% of faecal isolates (Table 3.3). It was found in one isolate from the Italian and Norwegian faecal samples (Table 3.4).

erm(E) was found in 5.7% of the faecal isolates (Table 3.3). It was absent in all the erythromycin resistant faecal isolates except two from the Italian samples (Table 3.4).

Hybridisation to other *erm* probes was observed in 14.3% of the total faecal isolates (Table 3.3). 25.7% of erythromycin resistant faecal isolates did not contain an *erm* gene that was probed for.

3.3.4 Vancomycin Resistance in the Aerobic / Facultative Anaerobic Cultivable Oral and Faecal Flora

Levels of vancomycin resistance in the Gram positive aerobic and facultative anaerobic cultivable portions of the oral and faecal samples are shown in Figure 3.5. However, as with the erythromycin plates no attempt was made to exclude the intrinsically resistant Gram negatives. No Gram positive vancomycin resistant isolates were found by Gram staining, thus the level of vancomycin resistance in the cultivable portions of the oral and faecal microbiota is considered to be zero in this study.

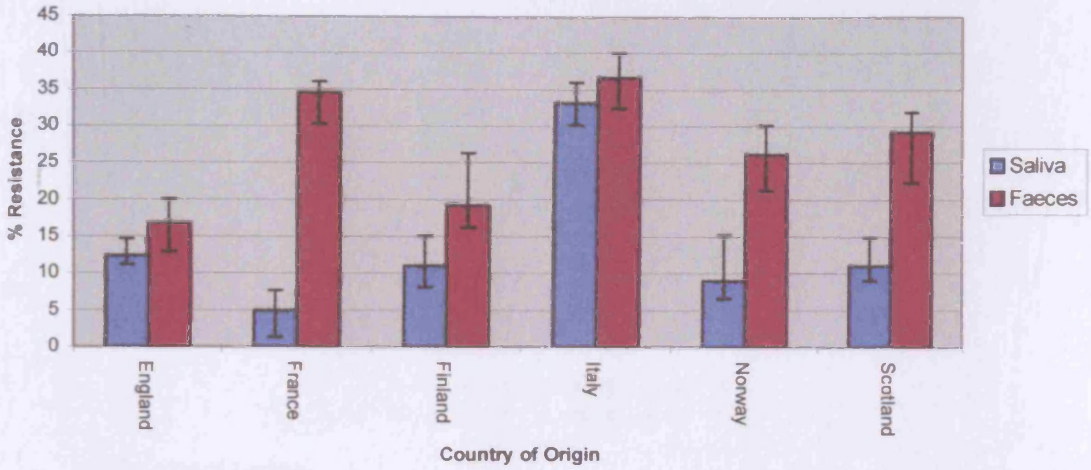


Figure 3.5: Vancomycin resistance profile of the oral and faecal aerobic / facultative anaerobic cultivable flora of six European countries.

3.3.5 Co-resistance

80.2% of tetracycline resistant isolates contained more than one *tet* gene. *tet*(M) and *tet*(O) occurred together in 59 isolates (55.1 % of total tetracycline resistant isolates). *tet*(M) occurred without *tet*(O) in 18/79 (22.8%) isolates and *tet*(O) occurred without *tet*(M) in 11/71 (15.5%) isolates.

tet(M) and the integrase, *int*, of Tn*916* occur together in 71 isolates (66.4 % of the total tetracycline resistant isolates), with *tet*(M) occurring in the absence of *int* in 8 isolates, and Tn*916 int* occurring without *tet*(M) in 1 isolate (Figure 3.6).

tet(M) was found with *erm*(B) in 55 isolates (44.7 % of total isolates). *tet*(M) occurred without *erm*(B) in 24/79 (30.4 %) isolates harbouring this determinant, *erm*(B) occurred without *tet*(M) in 12/69 (17.4 %) isolates.

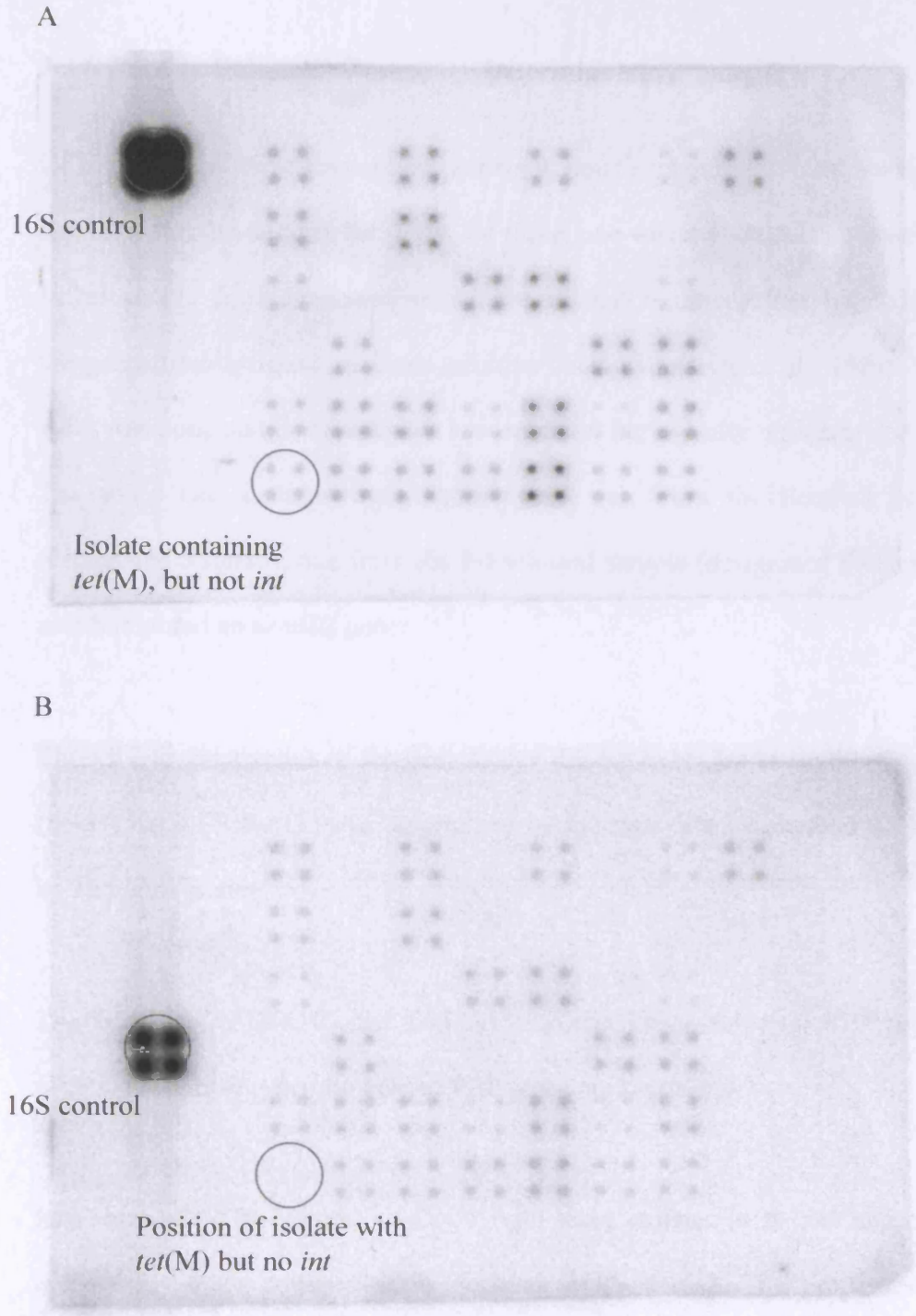


Figure 3.6: Macroarray results for the hybridisation of oral isolates with A). the *tet*(M) probe and B). the Tn*916* integrase probe. The isolates have been blotted and fixed to the nylon membrane, and the *tet*(M) and *int* probes hybridised. Illustrates the apparent linkage between the two genes in some cases.

3.3.6 Rare or Unknown Resistance Determinants

Of the Gram positive tetracycline resistant isolates identified, 3 did not hybridise to any of the probes used in the array. Of these, one was a *Pediococcus* from the Italian faecal sample. Most *Pediococcus* spp. are resistant to tetracycline, but do not harbour the genes usually found in Gram positive cocci (Tankovic et al., 1993). No further work was done on this isolate as it is considered intrinsically resistant.
The other two isolates were streptococci, one from the Scottish oral sample (designated SStet15), one from the French oral sample (designated FRStet12) which also harboured an *erm*(B) gene.

The MIC to tetracycline of the isolates that did not hybridise to anything on the array (SStet15 and FRStet12) was determined by the agar dilution method (Chapter 2) to be 32 μg/ml in air.

The PCR of the SStet15 and FRStet12 isolates using universal RPP primers was negative suggesting that no known RPP genes were present.

BAC and pDL278 libraries of DNA from these isolates in *E. coli* and *S. mutans*, respectively were constructed as detailed in Chapter 2. The properties of these libraries are shown in Table 3.5.

	FStet12 (*tet*(32))		SStet15		FRStet12	
	pCC1BAC	pDL278	pCC1BAC	pDL278	pCC1BAC	pDL278
Average insert size (Kb)	30	8	26	8	22	7
Size of library (Mb)	42.5	32.5	27.4	22.4	26.6	22.5
Coverage (%)	99	99	99	99	99	99

Table 3.5: Insert size and coverage (based on the calculation detailed in Chapter 2) of the libraries in *E. coli* and *S. mutans* of the isolates harbouring rare or unknown tetracycline resistance determinants.

Approximately 27 Mb of each isolate BAC library, and ~22.5 Mb of each isolate pDL278 library were screened on selective medium containing tetracycline at 10 μg/ml. No clones expressing tetracycline resistance were found.

3.4 Discussion:

The total cultivable flora of the oral and faecal microbiota has been investigated in previous studies (Kleessen, 2000; Sutter, 1984; Moore & Holdeman, 1974; Justesen *et al.*, 1984). The species present have been described in detail (Marsh, 2003; Paster *et al.*, 2001). In this study we have concentrated on the aerobic and facultative anaerobic cultivable flora due to restrictions in culture techniques available in the laboratory and as a result of the distance some of the samples have to travel before processing can occur. It is further refined to Gram positive bacteria.

3.4.1 Tetracycline and Erythromycin Resistance in the Aerobic / Facultative Anaerobic Oral Cultivable Flora

3.4.1.1 Levels of Resistance

Tetracycline and erythromycin resistance levels in the total cultivable flora from each European country were not found to be statistically different by the use of the students t-test. This suggests that resistance persists in the oral cultivable flora of healthy adults and does not vary according to geographic location. However, a range of levels of resistance was found.

In the English sample resistance levels are similar to the average levels found by Villedieu *et al.*, (2003) i.e. that 11 % of the oral cultivable flora were tetracycline resistant (cf 23.6 % for the English sample in this study). The differences are most likely due to the absence of obligate anaerobes in this study, however, normal population flux within the oral cavity, or sampling differences between the two studies may also contribute (Kolenbrander, 2000).

Other studies have been conducted on the prevalence of individual tetracycline resistance determinants in the oral cavity, however, these have been on isolates from

patients with endodontic problems (Rossi-Fedele *et al.*, 2006) and periodontitis (Lacroix & Walker, 1995, 1996), and so direct comparisons between the current study and these previous studies is not possible due to the changes in population composition brought about by the diseased state (Marsh, 1999, 2001) and the selective effect that any antimicrobial therapy may have had on the incidence and persistence of tetracycline- and erythromycin-resistant bacteria (Ready *et al.*, 2002; Edlund & Nord, 2000).

3.4.1.2 Composition of the Resistant Flora

In the oral cavity, in healthy adults, by far the most predominant species are the streptococci which constitute ~ 20% of the normal flora, at up to 10^6 cfu per ml saliva (Kohler, 1992). This is reflected in the results of the species identification of the resistant isolates taken from the oral samples in which streptococci predominate (74.7 % of oral isolates). This allows increased opportunities for these species to obtain resistance genes, furthermore, this genus contains naturally competent organisms (Stone & Kwaik, 1999; Solomon & Grossmann, 1996). Oral staphylococci and lactobacilli commonly have a high level of tetracycline and erythromycin resistance (Villedieu *et al.*, 2003, 2004) in agreement with this study. Other resistant genera isolated from the oral samples (Section 3.3.2) have all previously been reported in the oral cavity (M Roberts, 1999, 2002, 2004).

3.4.1.3 Genetic Basis of Tetracycline Resistance

3.4.1.3.1 *tet*(M)

In this study the most common tetracycline resistance determinant in the oral cavity was *tet*(M). This agrees with previous studies on both the cultivable and total oral

flora (Lancaster *et al.*, 2005; Villedieu *et al.*, 2003; Diaz-Torres *et al.*, 2006). It was found to occur in 76.8 % of the total tetracycline resistant oral isolates. It has previously been found to account for tetracycline resistance in 56 % of tetracycline resistant isolates from the oral cavity of healthy children (Lancaster *et al.*, 2003); and 79 % of resistant isolates from healthy adults (Villedieu *et al.*, 2003) illustrating that levels of *tet*(M) are relatively stable in the oral cavity of healthy adults. The wide-spread distribution of *tet*(M) is explained in part by its presence on Tn*916*-like elements, the integrase of which was commonly found in 89.9 % of isolates harbouring the gene. Tn*916*-like elements have been found in many oral species (Bentorcha *et al.*, 1992; McKay *et al.*, 1995; Mercer *et al.*, 2001) and *Tn916* has been shown to readily transfer among oral streptococci (Mercer *et al.*, 2001) and enterococci (Bentorcha *et al.*, 1992), and in microcosm dental plaques (Roberts *et al.*, 2001a). This is likely to account for the observed linkage (i.e. they are present in the same cell) of *tet*(M) and *int* in the macroarray results for the isolates. In addition, *tet*(M) is found on a range of other mobile elements (Table 1.7) which is likely to account for the occurrence of *tet*(M) without *int* in 8 isolates. Furthermore, Tn*916* has been found to harbour *tet*(S) in place of *tet*(M) (Tn*916*S) (Lancaster *et al.*, 2004); the ability of this family of elements to aquire different tetracycline resistance genes provides a plausible explanation for the occurrence of *int* without *tet*(M), an alternative is that it is harboured by a phenotpyicaly negative element.

tet(M) was harboured in the same isolate as *erm*(B) in 55 isolates. These genes have been reported together in a number of studies (Nielsen *et al.*, 2004; De Leener *et al.*, 2004; Betriu *et al.*, 2002), and have been shown to be linked on Tn*1545*-like elements and on the conjugative transposon Tn*3872*. This point is also relevant for the faecal isolates, and may explain why *tet*(M) and *erm*(B) are so common and why

they are often found in the same cell. Further work should include a the probing of a southern blot with a probe for *tet*(M), *erm*(B), the Tn*916/1545* integrase and the kanamycin resistance gene to establish whether the genes are linked on the same fragment of DNA, if they are they would likely be present on a Tn*1545*-like element as previously described (Villedieu *et al.*, 2004).

3.4.1.3.2 *tet*(O)

Villedieu *et al.*, 2003 found *tet*(O) to be common in the resistant flora, harboured by 10.4 % (mean) of tetracycline resistant isolates (the third most common determinant after *tet*(M) and *tet*(W) in their study). In this study, *tet*(O) is the second most commonly detected *tet* gene in the oral samples, found in 66.7 % of tetracycline resistant isolates. One possible explanation for this difference is the differences in breakpoints used. Villedieu *et al.*, (2003) used 8 μg/ml tetracycline based on the recommendations of the NCCLS (National Committee for Clinical Laboratory Standards. 1993), whereas in this study a lower breakpoint of 2 μg/ml tetracycline has been used as recommended by the BSAC (British Society for Antimicrobial Chemotherapy) (MacGowan & Wise, 2001; Andrews, 2001). Thus it is likely that this study would recover higher numbers of isolates. Furthermore, this study does not include strict anaerobes or Gram negatives therefore the results of the two studies are difficult to compare since the incidence some *tet* genes is different in aerobic and anaerobic organisms (reviewed in Levy *et al.*, 1989; Roberts, 1996).

The high incidence of *tet*(O) is probably due to its presence on mobile genetic elements, although only two have been characterised (*Campylobacter jejuni* plasmids pCC31 and pTet), however, these elements have not been shown to move outside the genus (Giovanetti *et al.*, 2003). In this study 80.4 % of *tet*(O) genes were found in

streptococci, which may play a large part in explaining its wide spread distribution due to the natural competence of some members of this genera (Stone & Kwaik, 1999; Soloman & Grossmann,1996).

In the current study *tet*(O) was found in *Actinomyces* spp. for the first time. Actinomycetes are implicated in infections of the tissue adjacent to dental implantation elements and tooth extraction wounds (Schaal *et al.*, 1992; Jeansonne, 2005), and tetracycline is one antibiotic used in the treatment of such infections (Bubbico *et al.*, 2004). This finding has important implications for therapeutic choices since tetracycline therapy may select for resistant *Acitnomyces* spp. harbouring this gene and cause the treatment to fail.

3.4.1.3.3 *tet*(W)

tet(W) was the third most commonly identified tetracycline resistance determinant in the oral flora in this study was identified in 10.1 % of the total tetracycline resistant bacteria compared to 21 % of isolates in the Villedieu study (2003). Again, differences may be due to the absence of obligate anaerobes in this study. *tet*(W) was first found in *Butyrivibrio fibrisolvens*, an anaerobe isolated from the rumen (Melville *et al.*, 2004). It has subsequently been found in *Bifidobacterium* sp. (Masco *et al.*, 2006), *Lactobacillus* sp. (Kastner *et al.*, 2006), and *Megasphaera* sp. (Stanton *et al.*, 2004) suggesting it is predominant among strict anaerobes (which are absent in this study). Furthermore, it has also been found in *Veillonella* spp., and *Neisseria* spp. (Villedieu *et al.*, 2003) however, Gram negative bacteria are excluded from this study. The Villedieu (2003) study also found *tet*(W) in *Bacillus* spp. and *Actinomyces* sp. which is mirrored in this study.

3.4.1.3.4 *tet*(32)

In other studies using the macroarray *tet*(32) has always hybridised with *tet*(O) (Andrea Patterson, personal communication, 2004), suggesting the presence of mosaic-type genes (Scott *et al.*, 2005). Such hybrid genes are composed of different sections of *tet*(O) and *tet*(32). In this study one of the streptococci isolated from the oral cavity was found to hybridise to the *tet*(32) probe, but not the *tet*(O). It is possible that this gene is the first example of a complete *tet*(32) isolated from the oral cavity.

The DNA from this isolate was cloned into both a Gram negative (*E. coli* in the pCC1BAC vector) and a Gram positive (*S. mutans* in pDL278) and screened for expression. ~36 Mb of the BAC library and 32.5 Mb of the pDL278 library was screened, which is equivalent of 17.7 streptococcal genomes (based on the *S. mutans* UA159 genome accession number NC_004350 (2.03 Mb)) for BAC and 15.9 streptococcal genomes for pDL278. No tetracycline resistant clones were found. There are various reasons why a gene might not express in a heterologous host (Gabor *et al.*, 2004). Bias in the cloning of the *tet*(32) encoding fragment may prevent successful cloning. The restriction sites used may be too far away from the gene and the large DNA fragment produced would clone or express less efficiently. However, if successfully cloned; the size of insert and the presence of expression signals that are functional in the host organism; and the correct folding of the resulting protein in a heterologous host by transacting host factors (chaperones, cofactors, protein-modifying enzymes) all contribute to the likelihood of successful expression (Gabor *et al.*, 2004). Chapter five details the successful cloning of *tet*(S) from *Enterococcus faecalis* and its failure to express in the EPI3000 *E. coli* host.

No clones containing *tet*(32) were found by probing the BAC library with a *tet*(32) derived PCR probe. ~48 Mb was screened this way (equivalent of 23.5 streptococcal genomes). This suggests the problem is due to an inability to clone the *tet*(32) sequence.

3.4.1.4 Genetic Basis of Erythromycin Resistance

3.4.1.4.1 *erm*(B)

In this study *erm*(B) was the most common *erm* (methylase) gene in the oral aerobic / facultative anaerobic cultivable flora. Most other studies that have investigated erythromycin resistance have found *mef*(A) to be the predominant erythromycin resistance gene in the cultivable oral flora (Villedieu *et al.*, 2004; Ojo *et al.*, 2004). However, these studies identify *erm*(B) as the most common *erm* gene. In the oral cavity it has previously been reported to be harboured by 33 % of erythromycin resistant cultivable isolates (Villedieu *et al.*, 2004) cf. 71.4 % in this study, however, as with the tetracycline breakpoint, the erythromycin breakpoint used in the study by Villedieu *et al.*, (2004) was higher: 4 μg/ml compared to 1 μg/ml in this study as advised by BSAC (MacGowan & Wise, 2001; Andrews, 2001).

In this study, the oral streptococci accounted for 86.0 % of *erm*(B)-harbouring isolates, compared to 93.9 % in the Villedieu *et al.*, study (2004), illustrating the relatively stable nature of *erm*(B) within streptococci, and high-lighting their importance as a reservoir of erythromycin resistance as has previously been described (Bryskier, 2002). The natural competence of this genus partly explains the high incidence of resistance genes it contains (Stone & Kwaik, 1999). Furthermore, as with the tetracycline resistance determinants, the erythromycin resistant

determinants are commonly found on mobile genetic elements (Table 1.6), including Tn*1545*-like transposons (Seral *et al.*, 2000), which goes some way to explaining their commonality in the oral cavity.

In this study *erm*(B) was found in *Rothia* sp. for the first time. *Rothia* spp. are common inhabitants of the human oral cavity and causative agents of serious dental infections including caries and infectious endocarditis (Boudewijns *et al.*, 2003). Although erythromycin is not used in the treatment of *Rothia* spp. infections, the presence of *erm*(B) on mobile elements may link it to other resistance determinants with potential detrimental effects on antibiotic therapy.

3.4.1.4.2 Other *erm* genes

This study identified *erm*(F) (a methylase gene) in 7.1 % of resistant isolates. Other studies have identified methylases as being common in the oral cavity of healthy humans (Ojo *et al.*, 2004; Villedieu *et al.*, 2004), but so far *erm*(F) has only been quantified in studies on samples of pathogens (Chung *et al.*, 2002). As with *erm*(B), *erm*(F) has been found on a number of mobile elements including CTnDOT, pBF4 and others (Table 1.6) (Whittle *et al.*, 2001; Shoemaker *et al.*, 1985) which is one of the causes of it being so widespread.

3.4.2 Tetracycline and Erythromycin Resistance in the Aerobic / Facultative Anaerobic Faecal Cultivable Flora

3.4.2.1 Composition of the Resistant Flora

The resistant faecal flora isolated in this study has a predominance of enterococci. This is probably due to the fact that the culture techniques used here were not

suitable for strict anaerobes, which out number aerobic bacteria by 100-1000:1 in the intestinal tract (Guarner & Malagelada, 2003). However, enterococci are commonly reported in the faecal flora in culture studies (Blaut *et al.*, 2002, Hayashi *et al.*, 2005), as are streptococci which were the second most common isolate from the faecal samples in this study and previous work (Blaut *et al.*, 2002).

3.4.2.2 Levels of Resistance

Many genera found in the gut harbour antibiotic resistance genes (Krause, 2002), and the gut has been the place where many novel genes have been found (Scott, 2002; Calva *et al.*, 1996; Osterblad *et al.*, 2000; Rice *et al.*, 2004). Unfortunately, it is difficult to compare the results of this study to previous studies on antibiotic resistance in the human gut as this study concentrates on the aerobic and facultative anaerobic species (due to sample collection and processing), whereas other studies include the obligate anaerobes including the Bacteroides / Prevotella group which constitute the majority of the cultivable human faecal flora (Upreti *et al.*, 2004; Blaut *et al.*, 2002).

Levels of tetracycline and erythromycin resistant bacteria show no significant difference, as determined by the student's t-test, between countries suggesting resistance among the Gram positive aerobic and facultative anaerobic cultivable flora of different countries is similar despite different levels of usage of antibiotics (Turnidge, 2001; Goossens *et al.*, 2005; Patrick *et al.*, 2004).

3.4.2.3 Genetic Basis of Tetracycline Resistance

3.4.2.3.1 *tet*(M)

tet(M) is the most common *tet* gene in the cultivable aerobic / facultative anaerobic isolates. As with isolates from the oral cavity, *tet*(M) was commonly found in the same bacterium as the Tn*916* integrase. This may be responsible for its transfer throughout the community, since it has been shown to transfer readily in the gut of gnotobiotic mice (Alpert *et al.*, 2003; Bahl *et al.*, 2004)

3.4.2.3.2 *tet*(O)

tet(O) is the second most common tet gene isolated. As with the oral *tet*(O) genes, their wide distribution in the faecal flora is likely to be due to the presence on mobile elements, including the *C. jejuni* plasmids pTet and pCC31, which are likely to be responsible for the previously observed transfer of *tet*(O) (Giovanetti *et al.*, 2003). *tet*(O) is common in the streptococcal isolates (their natural competence contributes to the high levels), and the enterococci. Enterococci have been found to exhibit high levels of tetracycline resistance (Hummel *et al.*, 2006; Bentorcha *et al.*, 1992) and have been shown to be viable recipients of numerous mobile elements (Klare *et al.*, 2001; Simjee & Gill, 1997). This may explain the high levels of resistance in enterococci in this study.

3.4.2.3.3 *tet*(Q)

tet(Q) is the third most isolated *tet* gene in this study. It was found in *Enterococcus* spp., *Lactobacillus* spp., and a *Streptococcus* spp. It has been previously found to be harboured by 80 % of Bacteroides strains found in the colon (Shoemaker *et al.*,

2001) in a study that implicated the conjugative transposon CTnDOT which has the ability to transfer to other genera (Shoemaker *et al.*, 2001) and may explain its predominance in the isolates of this study. As with *tet*(O), *tet*(Q) is common among enterococcal isolates, therefore the high levels of genetic exchange exhibited by this genus (Simjee & Gill, 1997) may explain why *tet*(Q) is so wide-spread.

3.4.2.4 Genetic Basis of Erythromycin Resistance

The most commonly isolated *erm* gene from the human faecal samples in this study was *erm*(B). As is the case with it's predominance in the oral samples, this is probably due to its presence on Tn*1545*-like transposons (Seral *et al.*, 2000). Furthermore, it has previously been found to be present in 100 % of human-derived enterococci (DeLeener, 2004), the most common genus isolated in this study. In this study it was harboured by 93.4 % of the tetracycline resistant enterococci.

3.4.3 Vancomycin Resistance

No vancomycin resistant Gram positive isolates were recovered during this study. Vancomycin resistance has been reported in various oral and faecal species, both Gram negative and Gram positive (Domingo *et al.*, 2005), however, they have not been isolated in studies similar to this (Bueris *et al.*, 2005; Ready *et al.*, 2003). It is possible that the normal microbiota of humans has not yet aquired vancomycin resistance in any great amount and as yet remains relative free from mobile elements carrying this resistance determinant.

3.4.4 SStet15 and FRStet12

Two oral streptococcal isolates did not hybridise to any of the probes on the array. These isolates harbour either rare tetracycline resistance determinants or previously unreported determinants. A PCR reaction using universal RPP primers was negative (Appendix 4), indicating that the tetracycline resistance genes in these isolates are not known RRP genes. The following efflux and enzymatic genes are not on the array: *tet*(K), *tet*(L), *tet*(V), *tet*(31), *tet*(33), *tet*(35), *tet*(38), *tet*(39), *tcr*3, *otr*(B) and *otr*(C) so may possibly be responsible for resistance in these strains. However, the MIC of both isolates was between 32 μg/ml – 64 μg/ml. Tetracycline efflux genes confer a low MIC (Webber & Piddock, 2003) therefore the probability of these streptococci harbouring efflux genes is relatively small.

It is possible that these isolates harboured one of the enzymatic inactivation genes (*tet*(X) or *tet*(37)); or *tet*(U) which confer resistance through an unknown mechanism (Roberts 2005). This would need to be verified by PCR using the appropriate control strains. In this study, the control strains were not available and single species libraries were made of SStet15 and FRStet12. These libraries were screened on selective agar. No resistant clones were detected when using an *E. coli* host (~27 Mb of each library was screened, corresponding to ~13.3 genomes of *S. mutans*), suggesting that there is a problem with expression of the gene in this host, however, when cloned into a streptococcal host this also yielded no resistant clones (~22.5 Mb of each library was screened (~11.1 streptococcal genomes)). As outlined above, there are numerous reasons why this may have occurred (Gabor *et al.*, 2004). It was not possible to probe the libraries as this requires knowledge of the gene sequence. Thus it is not possible to determine which resistance gene is responsible for tetracycline resistance in these isolates without sequencing the whole genome of the

original isolate or by using transposon mutagenesis to provide a tetracycline sensitive mutant and a platform from which to sequence out into the genome of the mutant strain. These strains have therefore been stored as glycerol stocks for future investigations.

3.4.5 Conclusions

There is no significant difference between the incidence of tetracycline and erythromycin resistance in the Gram positive aerobic / facultative anaerobic portion of the cultivable microbiota of six European countries.

The most common tetracycline resistance determinants in the oral isolates were *tet*(M), *tet*(O) and *tet*(W) (in decreasing order). In the faecal isolates *tet*(M), *tet*(O), and *tet*(Q) (in decreasing order) were the most common *tet* genes.

The most common erythromycin resistance determinants probed for were *erm*(B), *erm*(F) and *erm*(E) (in decreasing order) for both the oral and faecal isolates.

One isolate, FStet12, hybridised to the *tet*(32) probe, but not the *tet*(O) probe, suggesting it is a complete *tet*(32) gene or novel mosaic gene. BAC and pDL278 libraries were prepared, but no expression of tetracycline resistance was detected.
Two tetracycline resistance isolates did not hybridise to any of the probes on the array, therefore they contain either a rare *tet* gene or a novel resistance gene.
Libraries were made from DNA from the isolates but no tetracycline resistant clones were recovered. These strains are awaiting further investigation.

CHAPTER FOUR

Analysis of Metagenomic Libraries from Human Oral and Faecal Environments.

4.1 Introduction:

In the relatively well-studied microbiota of the human oral cavity, where it is estimated that there are more than 800 species of bacteria (William Wade, personal communication 2006), at least half of these are considered to be not yet cultivable (Kazor *et al.*, 2003; Pastor *et al.*, 2001). Likewise in the human gut approximately 20-40% (Blaut *et al.*, 2002; Gill *et al.*, 2006) of the estimated >1000 species are not yet able to be cultivated (reviewed in Kaper & Sperandio, 2005). One of the ways to obtain information about all of the organisms present in these complex communities is through the use of metagenomic approaches such as the construction of BAC libraries (Handelsman, 2004; Streit & Schmitz, 2004). Metagenomic studies have previously been used to investigate the healthy human faecal microbiota: Manichanh *et al.* (2006) compared the healthy microbiota to that of Crohn's patients. The predominant phyla in the healthy library were the Bacteroides and Firmicutes (of which *Clostridium* spp. predominated). All species identified were from these phyla or the Actinobacteria or Proteobacteria. The study also found 34 % of sequences were novel. Gill *et al.* (2006) employed a whole-genome shotgun sequencing approach to investigate diversity (identifying 16S rRNA sequences from random shotgun assemblies and supplementing the data with 16S rDNA libraries). The study also identified the Firmicutes division as predominant, and identified the Actinobacteria as the only other bacterial division present. The same study also used COG analysis (which groups functionally related genes using evolutionary relationships) to identify the functional diversity of the faecal microbiota. It found the microbiota enriched for genes involved in the metabolism of glycans, amino acids, and xenobiotics; methanogenesis; and 2-methyl-D-erythritol 4-phosphate

pathway-mediated biosynthesis of vitamins and isoprenoids (i.e. pathways not encoded by the human genome).

The healthy oral microbiota has yet to be characterised using metagenomic libraries, however, a 16S rRNA sequence approach has been used which found the predominant phyla to be the Proteobacteria, the Gram positives, the spirochetes or the flavobacter-bacteroides group (M Wang *et al.*, 2005). The tetracycline resistant portion of the oral microbial metagenome has been investigated using metagenomic libraries by Diaz-Torres *et al.*, (2006) and was found to be predominated by enterococci, *Prevotella* spp., *Campylobacter* spp., and staphylococci.

This part of study reports the methods used for construction of BAC libraries from the human oral plaque and saliva and human faecal metagenomes. To determine if the libraries contained DNA derived from aerobes and anaerobes, and cultivables and not-yet-cultivables a random collection of cloned inserts were analysed. Using an end-sequencing approach and Blastn analysis on over 600 randomly selected clones, the current study demonstrates that the BAC libraries contain what would be expected of these two environments. In addition sequences were analysed using Blastx to investigate the range of putative proteins that could be encoded by the cloned DNA.

4.2 Materials and Methods

4.2.1 Fragmentation of DNA from Human Samples.

4.2.1.1 Mechanical Shearing

1 ml genomic DNA (between 60-100 µg/ml depending on country of origin) was sheared in a Hydroshear device (Genemachines, USA) which uses hydrodynamic shearing forces to fragment DNA. A shearing setting of 40 (arbitrary units of the machine) was used. 50 % of the sample under went 1 cycle of shearing, the other 50 % under went 5 cycles.

4.2.1.2 Fragmentation by Restriction Digest

50 µl aliquots of genomic DNA (diluted to ~50 µg/ml) were partially digested with *Sau3*A1 (multicore buffer; 2 units / 100 ng DNA) or *Hin*dIII (3.2 units / 100 ng DNA). In all cases digests were performed at 37 °C with 50 % of the sample digested for 1 min and 50 % for 3 mins. The reactions were subject to an ethanol precipitation and resuspended in 100 µl sterile dH_2O at 4 °C overnight.

All fragmented DNAs were run out on agarose gels (2 %) overnight at 30 V in a cold room (4 °C). Low Melting Point (LMP) agarose was used if DNA was to be extracted by agarase digestion

4.2.2 Extraction of DNA from Agarose Gels

4.2.2.1 Agarase Digestion

Gel slices containing HMW DNA were cut from the gels (above 40 Kb according to a lambda mono-cut molecular weight standard). They were melted at 65 °C and digested with agarase (1unit / 200 mg gel; 42 °C) until the gel had liquefied.

4.2.2.2 Gel Extraction using the Qiagen Gel Extraction Kit

Gel slices containing HMW DNA were cut from the gels and were subjected to DNA extraction using the Qiagen Gel Extraction Kit.

4.2.2.3 Electroelution

Gel slices containing HMW DNA were cut from the gels. They were placed in dialysis tubing prepared as described by the manufacturer. 250 µl sterile d H_20 were added to the gel slice in the tubing. The ends of the tubing were sealed with sterile metal clamps, and the tubing placed in a Bio-Rad gel tank in a cold room (4 °C). 25 V was applied for 1 hour. The tubing was removed and the DNA solution removed with a wide-bore pipette.

4.3 Results:

4.3.1 Fragmentation and Isolation of HMW DNA

The fragmentation of metagenomic DNA using the hydroshear machine was compared to the use of partial digests using a 4 bp cutter (*Sau3*AI) and a 6 bp cutter (*Hin*dIII) (Figure 4.1). The hydroshear was only capable of producing DNAs of up to ~40 Kb (Figure 4.2) compared with up to ~100 Kb (clone IStetC2) using the restriction digests (based on insert sizes determined by *Eco*R1 digestion of 20 or more clones). *Hin*dIII was found to be preferable to *Sau*3AI since it produced larger DNA fragments under the conditions tested.

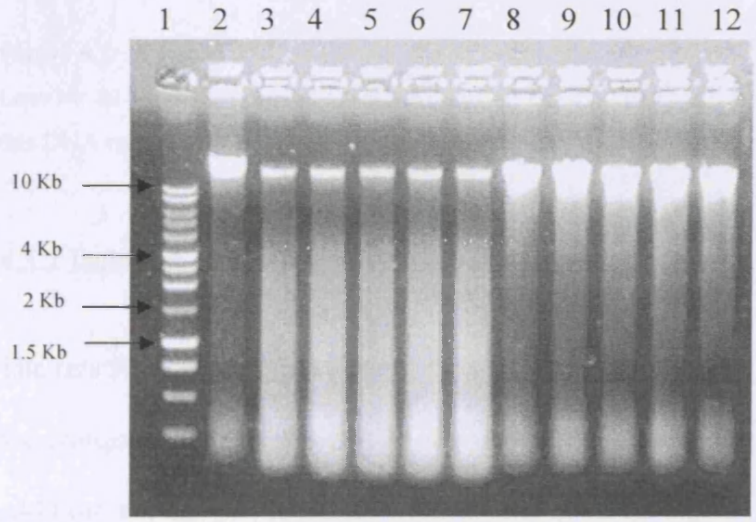


Figure 4.1: Genomic DNA (lane 2) digested with *Sau*3AI (lanes3-7) and *Hin*dIII (lanes 8-12). Digests for each enzyme had incubation times of 1, 2, 3, 4 and 5 mins left-right. Lane 1 is a 10 Kb marker.

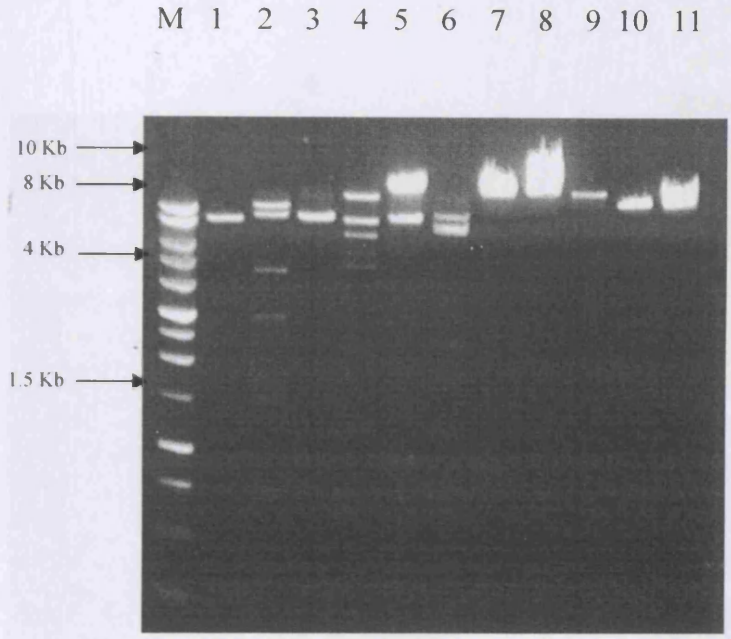


Figure 4.2: Digested BAC clones made using DNA fragmented using the hydroshear machine. Lane M: 10 Kb marker. Lanes 1-11 digested BAC clones. The average size of clone constructed using this DNA was 13 Kb. DNA was digested with *Eco*R1.

4.3.2 Isolation of HMW DNA from Agarose Gels

The relative yields of DNA recovery using electroelution, agarase and the Qiagen kit are compared in Figure 4.3. All methods resulted in a loss of DNA of >=70 %. In addition, the Qiagen kit was found to shear the DNA to ~10-20 Kb, and in all cases LMW DNA remained in the sample. Therefore, no size selection was made on the fragmented genomic DNA.

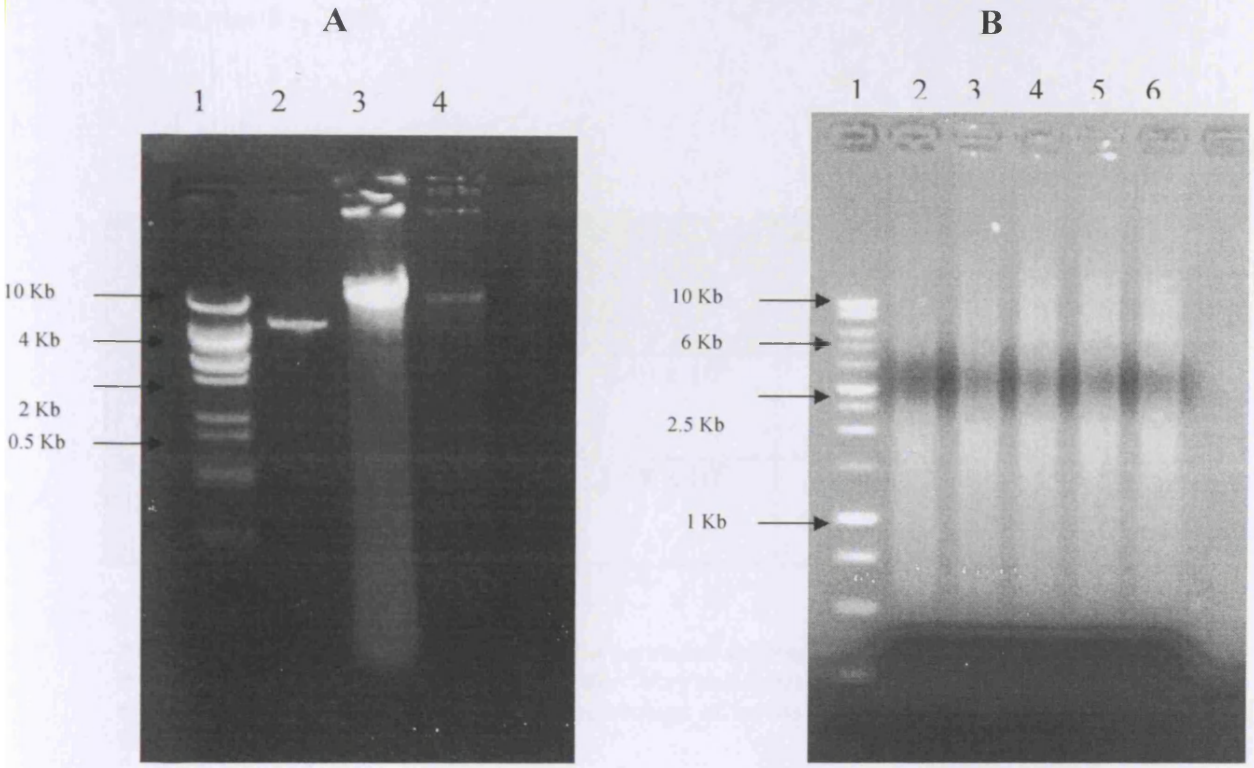


Figure 4.3: A. DNA recovered after size selection using electroelution and agarose digestion. Lane 1; 10 Kb marker. Lane 2; DNA recovered after electroelution. Lane 3; undigested genomic DNA. Lane 4; DNA recovered after agarase digestion.
B. DNA recovered using the Gel Extraction Qiagen kit (lanes 2-6). 10 Kb marker lane 1. Recovered DNA lanes 2-6 (performed 5 times on the same sample).

4.3.3 Library Construction

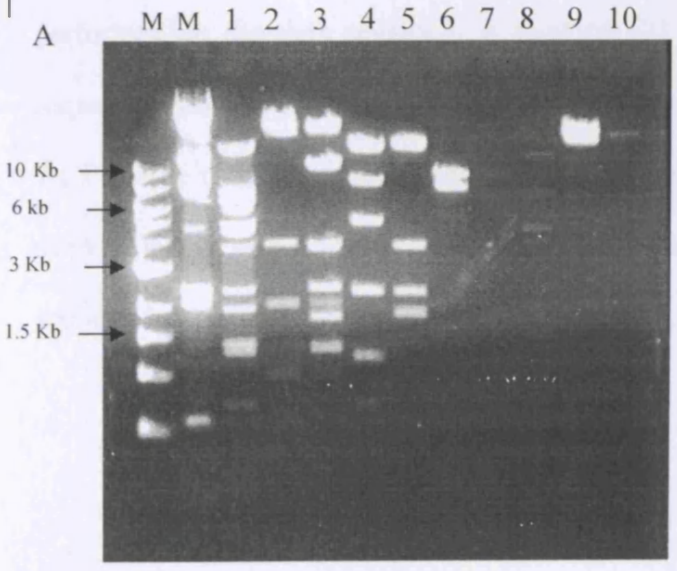
BAC libraries from each of the European Institutes were constructed, however for the purposes of this Chapter only the English libraries, one oral and one faecal, were analysed by end sequencing.

The average insert size for the English saliva library is 30 Kb, that for the faecal library is 24 Kb (Table 4.1), as determined by the restriction digest of 10 randomly

selected clones from each library (Figure 4.4). The range of insert size in these 20 clones was 8 – 70kb.

Sample	Average insert size (Kb)	Number of Clones	Size of Library (Gb)	Estimated Coverage of Microbiota[a]
English saliva	30	1.49×10^6	44.8	85%
English faecal	24	1.99×10^6	47.7	91%

Table 4.1: Large insert BAC libraries. The estimated coverage of the microbiota is the probability that any one sequence has been cloned into the library and is based on the equation detailed in Chapter two. [a]This Figure takes into account the percentage of inserts shown to contain human DNA (see below).



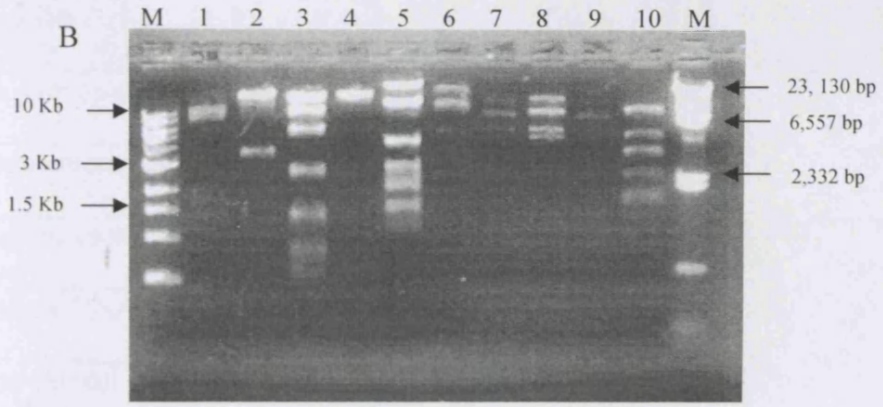


Figure 4.4: Sizing the inserts of the BAC libraries A). *Hin*dIII digested English saliva clones (lanes 1 – 10). Lanes M: 10 kb marker, *Hin*dII digested lambda. B). *Eco*RI digested Norwegian faecal clones (lanes 1 – 10). First lane M: 10 kb marker, second lane M: *Hin*dIII digested lambda.

4.3.4 Analysis of BAC Library Inserts

Randomly selected BAC clones from each of the libraries were subject to end sequencing (carried out at the Sanger Institute), and blastn and blastx searches were performed on the data obtained. A total of 621 sequences produced 327.6 kb of sequence information: 195.5 kb (336 sequences) from the saliva library (Appendix 7), 132.1 kb (285 sequences) from the faecal library (Appendix 8) (Table 4.2). The average length of read for the saliva end-sequences is 585.0 bp, for the faecal end-sequences it is 463.4 bp.

Library	English Saliva	%	English Faecal	%	Total
No. clones end-sequenced	336		285		621
Total amount of DNA sequenced (Kb)	195.5		132.1		327.6
Sequences with BLASTX similarities <1e15	140	62.8	112	39.3	
Human DNA	80	44.9	0	0	
Non-human sequences with BLASTX similarities <1e15:	60	17.9	112	39.3	
Bacterial DNA	58/60	96.7	111/112	99.1	
Eukaryotic (non-human) DNA	1/60	1.7	1/112	0.9	
Viral DNA	1/60	1.7	0/112	0	
BLASTX no homology (below <1e15)	196	58.3	173	60.7	
Of which show homology to human DNA at the nucleotide level (BLASTN <1e15)	125/196	63.8 (37.2 % of total)	0	0	
Of which show homology to other eukaryotic / prokaryotic / viral sequences (BLASTN <1e15)	0	0	5/173 all bacterial	2.9 (1.8 % of total)	
Of which show no homology to sequences in the database (BLASTN <1e15)	71	21.1	168	58.9	
Total number of human sequences in the library	205	61	0	0	

Table 4.2: Analysis of End-Sequences of BAC Inserts.

A large amount of human DNA is contained in the saliva library but none was detected in the faecal library. The saliva library contains 61.0 % human DNA. Taking this into account, the coverage of the microbiota by each library has been calculated (Table 4.1).

4.3.5 G+C Analysis

As a further indicator of the diversity of cloned sequences the G+C content of the end-sequences of each library were calculated (Figure 4.5). The saliva library contained sequences with an average G+C content of 41.5% (26–65%), with its most common range being that between 35-39 %. In contrast, the faecal library has an average G+C content of 50.1% (29-71%). The most common faecal G+C range was between 45-49%.

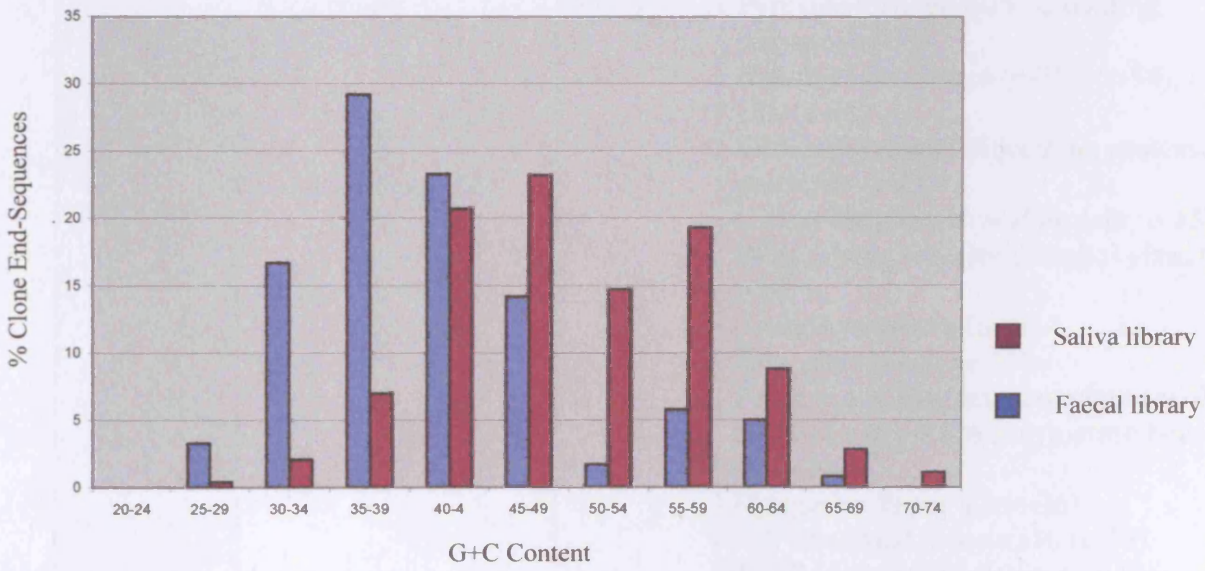


Figure 4.5: G+C Content of the Oral and Faecal BAC Library Inserts.

4.3.6 Translated End-Sequence Analysis

Blastx searches demonstrated that the cloned DNA had the potential to encode proteins predicted to be involved in a wide range of functions including central metabolism, response regulators, intercellular signalling, virulence, antibiotic

resistance and gene transfer (Table 3.3 and 3.4). The cut off value for acceptance of likely homologues was <1e15. This Figure was chosen because it is the more accurate of cut-off levels used in previous studies using blastx as a measure of diversity (Treusch *et al.*, 2004; Abascal & Valencia, 2002; Wintero *et al.*, 1996).

	Genus to which the closest matching protein belongs	**No. matching end-sequences**	**Functions**
Bacteria			
	Bacteroides	15	Pyruvate formate-lyase activating enzyme (e-37) Hypothetical protein (e-35), (e-54), (e-18), (e-22) Carboxy-terminal processing protease precursor (e-73) Conserved hypothetical protein (e-45) Biosynthetic arginine decarboxylase (e-104) Alanine racemase (e-80) Beta-glucosidase (e-43) Putative arginine decarboxylase (e-106) DNA-directed RNA polymerase beta chain (e-58) Elongation factor TS (e-36) 30S ribosomal protein s10 (e-33) dGTP triphosphohydrolase (e-49)
	Haemophilus	7	Large exoprotein involved in haem utilisation (e-92) Serine/threonine kinase (e-64) Prephenate dehydrogenase (e-110) Uncharacterised protein involved in chromosome partitioning (e-118) Hypothetical protein (e-42) Dexgoctulsonic acid synthetase (e-45) Molybdopterin converting factor subunit (e-78)

	Streptococcus	6	Hypothetical protein (e-19) Sortase (e-27) Site-specific recombinase (e-80) Formate acetyltransferase (e-103), (e-130) Putative histidine kinase of ComD (e-23)
	Clostridium	3	Pyridoxal kinase (e-22) Transketolase N-terminal (e-60) FAD synthase (e-26)
	Porphyromonas	3	Hydrolase (e-48) Hypothetical protein (e-19) Reverse transcriptase homolog (e-64)
	Treponema	3	Phosphoglycerate mutase (e-33) Ankyrin repeat protein (e-54) ABC transporter, ATP binding protein troB (e-20)
	Bacillus	2	Endo-beta-N-acetylglucosaminidase (e-16) Type-I restriction modification system restriction subunit (e-70)
	Desulfitobacteria	2	Preprotein translocase (e-37) ABC-type transport system ATPase component (e-71)
	Kineococcus	2	Translation elongation factor (e-32) Predicted membrane protein (e-16)
	Moorella	2	Guanosine polyphosphate pyrophosphohydrolase (e-28) Leucyl-tRNA synthetase (e-84)
	Pasteurella	2	Unknown hypothetical protein (e-20) PyrD dihydrorotate dehydrogenase (e-51)
	Actinobacteria	1	Geramylgeranyl phosphate synthase (e-72)
	Azotobacter	1	ATPase (e-52)
	Corynebacteria	1	Conserved hypothetical protein (e-68)
	Fusobacteria	1	Hypothetical exported protein (e-36)
	Mycobacterium	1	Phosphotransacetylase (e-35)
	Mycoplasma	1	Conserved hypothetical transmembrane protein (e-15)
	Phytoplasma	1	Nef attachable protein (e-15)
	Streptomyces	1	Conserved hypothetical protein (-69)
	Sulfurospirillium	1	Formate dehydrogenase subunit (e-60)
	Thermosynechococcus	1	Heat shock protein HSP33 (e-34)
	Xanthomonas	1	Branched chain amino acid aminotransferase (e-54)

Eukaryotes	*Dictyostelium*	1	Hypothetical protein (e-30)
Viral			
	MS-associated retrovirus	1	Hypothetical protein (e-40)
No Significant Homology		71	

Table 4.3: Translated End-Sequence Analysis of Oral BAC Clones. Only values of <1e15 were accepted.

	Genus to which the closest matching protein belongs	No. matching end-sequences	Function
Bacteria			
	Clostridium	36	Serine threonine phosphatase (e-21) Pyruvate phosphatase dikinase (e-18) (e-15) Gyrase B (e-15) RNA polymerase beta-subunit (e-92) DNA-directed RNA polymerase (e-99) SecA protein (e-42), (e-30) L-lactate dehydrogenase (e-59) ABC-type multidrug/protein/lipid transport protein (e-30) Hypothetical protein (e-49), (e-48), (e-30), (e-46) Glycosyl hyrolase (e-17) R-phenyllactate dehydratase (e-29) Ferrous ion transport protein (e-64) Helix-turn-helix AraC type protein (e-21) Multidrug resistance protein (e-18), (e-26) Poly(A) polymerase (e-45) DNA topoisomerase (e-32) Glycogen synthase (e-73) Tyrosyl-tRNA synthetase (e-59) NAD/FAD binding protein (e-18), (e-15) 50S ribosomal protein (e-28), (e-27) ATPase central region (e-63), (e-68) Methyltransferase (e-58), (e-48), (e-58), (e-48) Anaerobic dicarboxylate transport protein (e-35) Carbamoyl-phosphate synthase (e-77)

	Bacteroides	15	Preprotein translocase (e-41), (e-101) Probable cation efflux pump (e-48) Putative sulfatase (e-44) Conserved hypothetical protein (e-31), (e-71) ATP synthase beta-subunit (e-47) Phosphoribosylformylglucinamidine (e-126), (e-127) Outer membrane efflux protein (e-19), (e-31) Transcriptional regulator (e-18) Electron transport complex protein (e-33) Cu2+ homeostasis protein (e-47) Histidinol-phosphate aminotransferase (e-64)
	Campylobacter	7	Mob/transferase protein (e-99) TnpV (e-21), (e-29), (e-24), (e-27), (e-32), (e-32)
	Arthrobacter	6	Hypothetical protein (e-23), (e-23), (e-23), (e-23), (e-23), (e-23)
	Treponema	5	ORFA (e-77), (e-75), (e-76) Replication protein (e-21), (e-20)
	Enterococcus	4	Hypothetical protein (e-27), (e-26), (e-21) Prolipoprotein diacylgylceryl transferase (e-29), (e-27)
	Bacillus	3	Alkaline phosphatase synthesis response regulator (e-49) ATP-dependent RNA helicase (e-16) Alkaline phosphatase 2-component response regulator (e-25)
	Exiguobacterium	3	Peptide chain release factor (e-38), (e-34), (e-41)
	Lactobacillus	3	Putative carbohydrate kinase (e-48) Carbamoyl-phosphate (e-66) ABC-type antimicrobial peptide transferase (e-34)
	Caldicellulosirup tor	2	Metal dependent phosphohydrolase (e-15) Acetolactate synthase (e-24)
	Desulfitobacteriu m	2	Drug antiporter (e-56) ATP-binidng region (e-18)
	Mesorhizobium	2	Virulence factor SrfC homologue (e-45), (e-52)
	Staphylococcus	2	Replication protein (e-53), (e-56)
	Streptococcus	2	30S ribosomal protein SI (e-19) Site-specific recombinase (e-70)

	Symbiobacterium	2	Conserved hypothetical protein (e-24) Haloacid dehydrogenase (e-18)
	Syntrophomonas	2	LuxR regulatory protein (e-20) Pro-δ-E processing factor (e-20)
	Actinobacillus	1	Beta-galactosidase (e-49)
	Agrobacterium	1	50S ribosomal protein (e-21)
	Alkaliphilus	1	Nitric oxide reductase (e-27)
	Bifidobacterium	1	Beta-galactosidase (e-19)
	Butyrivibrio	1	Hypothetical protein (e-90)
	Crocosphaera (Synechocystis)	1	Cysteine desulphurase (e-34)
	Geobacillus	1	Trigger factor (e-23)
	Moorella	1	Sodium/sulphate symporter (e-58)
	Natronomonas	1	Molybdenum cofactor biosynthesis protein (e-28)
	Prevotella	1	Mobilisation protein (e-62)
	Porphyromonas	1	Hypothetical protein (e-15)
	Rhodoferax	1	Ferrireducans (e-24)
	Salmonella	1	LacZ fusion protein (e-30)
	Thermoanaerobacterium	1	Sodium/alanine symporter (e-48)
	Xanthomonas	1	Hypothetical protein (e-56)
Eukaryotes			
	Arabidopsis	1	Amino acid binding protein (e-30)
No Significant Homology		169	

Table 4.4: Translated End-Sequence Analysis of Faecal BAC Clones. Only values of <1e15 were accepted.

4.3.7 Analysis of TnpV Encoding End-Sequences

The spread of antibiotic resistance is mediated by the transfer of mobile genetic elements which harbour these genes (Cooper *et al.*, 1996; Salyers *et al.*, 2004). The end-sequence analysis of the English libraries revealed the relatively high incidence of the *tnpV* gene which is harboured by the mobilisable transposon Tn*4451* which also harbours the *catP* gene for chloramphenicol resistance (Lyras *et al.*, 2004). *tnpV* has also been found downstream of *tet*(O) in a *B. fibrisolvens* isolate (Scott 2002). The TnpV sequences were analysed further to determine if they were likely to have

the same phylogenetic origin which would be indicative of horizontal spread, and as a potential indicator of the spread of antibiotic resistance due to its association with resistance genes.

Six end-sequences in the faecal library showed homology to TnpV. These were aligned with known sequences of the protein, and a cladogram constructed (Figure 4.6).

The Cladogram shows that BACTnpV1, BACTnpV3 and BACTnpV6 are closely related, and BACTnpV2, BACTnpV4 and BACTnpV5 are also closely related.

They are all distantly related to the protein products of the *tnpV* genes in the database. In fact, they form a specific group, which suggests that they share a common ancestor (indicated by the arrow on Figure 4.6) that had diverged from the common ancestor of all the previously reported TnpV proteins.

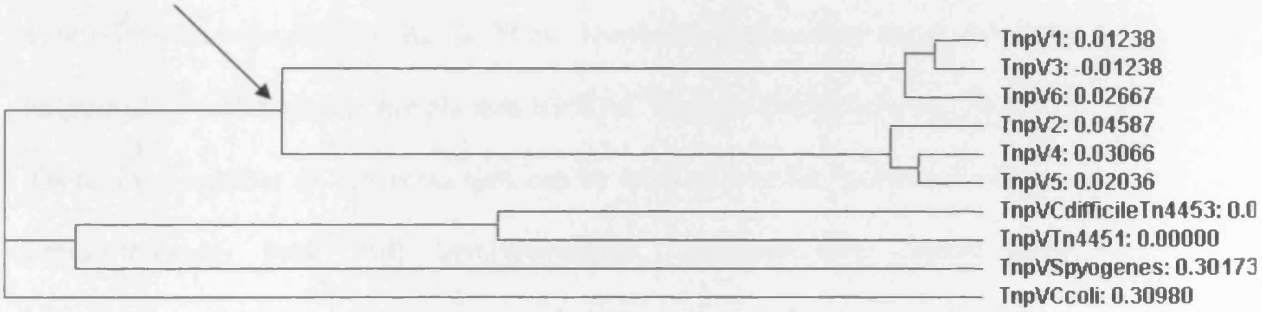


Figure 4.6: Cladogram showing the phylogenetic relationship between translated *tnpV* genes. Number of common ancestors can be determined by the number of nodes that separate genes, distances are listed beside the protein. The arrow represents the theoretical common ancestor of all the BAC derived TnpV sequences.

4.4 Discussion

4.4.1 Construction of HMW Libraries

To realise the full potential of the BAC system it is imperative to isolate high quality HMW DNA from bacterial cultures or environmental samples (Berry *et al.*, 2003; Strong *et al.*, 1997). The process of obtaining the DNA can be complicated if using environmental samples. Extraction of DNA from soils and faecal samples is especially problematic due to the potential contamination by organic compounds such as humic acids, which inhibit downstream molecular processes such as PCR and cloning (Tebbe & Vahjen, 1993; Trevors, 1996), and the difficulty in obtaining sufficient yield due to the adsorption of DNA to particulate surfaces (Krsek & Wellington, 1999).

A number of methods have been optimised for the removal of DNA from human and environmental samples for use in PCR. However, these often shear the DNA to lengths of ~10 kb which is insufficient for BAC libraries (McOrist *et al.*, 2002).

There are a number of processes that can be used to lyze the cells within a sample. Freeze-thawing, bead mill homogenisation, ultrasonication, liquid nitrogen, lysozyme and SDS in conjunction with chelating agents (EDTA) to inhibit nucleases (Miller, 1999; Krsek & Wellington, 1999), however, these too inflict mechanical shearing. As a result specialist methods have been developed. Zhou *et al.* (1996) first developed a method to extract DNAs of >23 kb by lysis with a high-salt extraction buffer, extended heating in the presence of SDS, hexadecyltrimethylammonium bromide and proteinase K to lyse the cell. However, Berry *et al.*, 2003 has since optimised extraction of HMW DNA using a combination of methods including the lysis of cells within biomass immobilised in agarose plugs, followed by pulsed-field

gel electrophoresis to isolate DNAs of the required size. The BAC libraries in the current study were constructed using HMW DNA extracted from the oral and faecal samples using a commercial kit. This produced DNAs of up to ~100Kb which was deemed sufficient for this project.

The use of partial digestion to fragment the DNA allows the possible inclusion of all sequences in the library. Although digestion introduces a bias in to the DNA cloned, compared to the use of mechanical shearing, it was the method of choice in the current study since it produced higher molecular weight DNA fragments. Furthermore, the cloning of sticky-ended DNA molecules is more efficient than the cloning of blunt-end fragments (which would be produced following mechanical shearing) (Maniatis, 1989).

Pulsed field gel electrophoresis (PFGE) is commonly used to isolate large DNA fragments for use in cloning thus maximising the efficiency of the BAC system (Strong *et al.*, 1997). The DNA is subsequently removed from the gel through the digestion of the agarose matrix by DNase-free agarase, or by electroelution whereby the DNA-containing gel slice is placed in dialysis tubing and a current applied to cause the DNA to migrate into solution (Osoegawa *et al.*, 1998). Both methods prevent unnecessary shearing of the DNA, although electroelution is markedly more efficient than agarose digestion (Strong *et al.*, 1997). No size selection of inserts was performed in this study due to the low efficiency of gel extraction and the finite amount of DNA available. It also prevents the introduction of another bias into the cloning process, therefore the insert sizes reported here are not the maximum permitted in BAC vectors (Shizuya *et al.*, 1992). This study is concerned with screening the library for functional antibiotic resistance genes, therefore, although a

greater number of clones would need to be screened in order to represent a good coverage of the microbiota, plating is not especially time consuming.

4.4.2 Blast Analysis

For the Blast analysis of the BAC end-sequences the cut-off of <1e15 was used. This figure was chosen as it is the most accurate cut off value used in previous studies of this kind (Treusch *et al.*, 2004). Blast analysis revealed that the libraries obtained from the oral sample contain a considerable amount of human DNA (61.0 %). This is not surprising as no effort was made to remove eukaryotic cells. The presence of human DNA further increases the number of clones that need to be screened to give an adequate coverage of the microbial metagenome. However, as discussed above if using a functional screen it is not a problem since only those clones containing the viable pathway or conferring the desired phenotype, such as antibiotic resistance would survive on selective media. Clones should, however, be analysed further to ensure the insert is of bacterial origin since Diaz-Torres *et al.*, (2006) found that some human DNA sequences can confer resistance to tetracycline via an unknown mechanism(s).

4.4.3 Putative Origins of Bacterial Inserts in the BAC Libraries.

The Blastx analysis of the BAC end-sequences gives an indication of the closest matches to the sequences obtained (Altschul *et al.*, 1990). Of the proteins identified few are phylogenetic markers, and previous studies have shown that protein encoding sequences are as predictive of genera as rRNA sequences in 57-96% of genes (Nesbo *et al.*, 2005). Therefore the results are more indicative than definitive.

4.4.3.1 Oral Library

The BAC clones containing bacterial inserts sequenced from the oral library show that the three most commonly matched genera, in homology searches, was *Bacteroides* followed by *Haemophilus* and *Streptococcus* (Table 4.3). This is in agreement with previous studies which have demonstrated the presence of these genera in the healthy oral cavity (Marsh, 1999, 2003; Tanner *et al.*, 2000; Paster *et al.*, 2001). In comparison, streptococci predominated in the culture study due to the fact that *Bacteroides* spp. are anaerobic. Furthermore, DNA from anaerobes makes up 72.2 % (74.8 % of faecal; 67.2 % of oral) of the DNA sequenced, thus indicating the libraries are representative of the organisms missed since the current culture study did not include obligate anaerobes.

This study has also found clones that can encode proteins homologous to those from bacteria not usually found in the oral cavity such as *Moorella* (at up to e-84), *Sulfurospirillium* (e-60), and *Thermosynechococcus* (e-34). *Moorella* species are anaerobes most commonly isolated from soil samples (Drake & Daniel, 2004). *Sulfurospirillium* are anaerobic, halorespiring bacteria associated with hot springs (Kamekura *et al.*, 1998), and *Thermosynechococci* are unicellular cyanobacteria which have been found in soils and lake sediments (Kucho *et al.*, 2004). Their presence here as sequences with a high similarity to those in the databases (hits of <1e15), prove that it is possible to clone DNA originating from species which are likely to be transient in the oral cavity. It also suggests that there is a possibility that the DNA from these transient organisms is capable of persisting in this environment for a period sufficient for transforming competent oral bacteria such as the

streptococci and *Neisseria* (Stone & Kwaik, 1999; Soloman & Grosmann, 1996). An alternative explanation for the isolation of DNA showing homology to these bacteria is that there are members of this genus present in the oral cavity which are yet to be identified and characterised.

Other interesting matches from the oral BAC clones include *Phytoplasma* which is the etiological agent of a number of plant diseases (Christensen *et al.*, 2005). It resides on the phloem sieve plates of plants, thus if a diseased plant was ingested the mechanical grinding of the stems by teeth would release the bacterium into the oral cavity. DNA related to that from *Mycoplasma* spp. was also found in the oral library. These are ubiquitous in nature and could similarly be ingested on plant matter (Rottem, 2003).

4.4.3.2 Faecal Library

DNA with homology to organisms previously reported in the human faecal microbiota are most commonly observed (Table 4.4), with mostly bacterial DNA being cloned. *Clostridium* spp., *Bacteroides* spp., and *Campylobacter* spp.. These have been reported to constitute up to 95% of the cultivable flora using a 16S rRNA gene amplification study (Blaut *et al.*, 2002; Gill *et al.*, 2006) and here represent 46.5% of sequences with homology to bacteria, with respective matches for each of up to e-99, e-127, and e-99. In contrast to this study, *Campylobacter* spp. were not found in the metagenomic library representing the healthy gut in the Manichanh study (2006), but the other two genera were predominant.

In contrast to the current culture study on the same samples (Chapter 3), the inserts were predominated by sequences with homology to obligately anaerobic genera (*Clostridium* spp., *Bacteroides* spp, *Treponema* spp., *Bifidobacterium* spp., and *Butyrivibrio* spp. amongst others), demonstrating the ability of the metagenomic approach to investigate a representative spectrum of the microbiota. Previous studies have reported these genera to be normal constituents of the microbiota (Gill *et al.*, 2006; Blaut *et al.*, 2002), however, Manichanh *et al.*, (2006) did not report these other genera in the metagenomic library constructed from faecal samples from a healthy volunteer.

In addition to the above genera, the current study also identified *Lactobacillus* and *Bifidobacterium*, which are considered commensal and are also taken as probiotics (Fooks & Gibson, 2002), and *Enterococcus*, *Bacillus*, *Staphylococcus*, and *Salmonella*, all of which are represented above the 1e15 cut-off level. They have all been previously reported in the faecal microbiota (Blaut *et al.*, 2002).

In this study only *Lactobacillus* spp. and *Staphylococcus* spp. were cultured from the same sample.

As with the oral library some matches were unexpected. Genera more commonly associated with the oral cavity were reported (*Treponema*, *Actinobacillus*) (Marsh, 1999, 2003). Only 29/500 oral species have been reported in faecal samples (Moore, 1994), however, it is possible that DNA may persist through the GI tract which may then have the potential to be cloned. Furthermore, some genera were found which have not previously been reported in the faecal microbiota. *Geobacillus*, an aerobic, motile Gram positive rod (Nazina *et al.*, 2001), *Caldicellulosiruptor* (Huang *et al.*, 1998), an anaerobic Gram negative rod, and *Alkaliphilus* are all more commonly

associated with soils and sediments (Takai *et al.*, 2005). *Xanthomonas*, known to cause Black rot in plants (Qian *et al.*, 2005) may be part of the transient microflora. It is possible that these organisms have entered the GI tract via the consumption of plant matter (which may harbour residual soil particles). However, it is also possible that these species are residents of the faecal microbiota and have yet to be cultivated.

4.4.3.3 Sequences With No Homology to Sequences in the Databases

Of the non-human sequences identified, in the oral cavity 56.8% of clones, and 60.7% of faecal sequences showed no significant similarity to anything in the databases. These Figures roughly reflect the predicted proportions of uncultured organisms present in each environment (Kazor *et al.*, 2003; Suau *et al.*, 1999), and further serves to indicate the libraries to be representative of the predicted communities. In comparison, the diversity study by Gill *et al.*, (2006) using 16S rDNA libraries and shotgun sequence assemblies containing 16S rRNA genes found only 23 % of sequences were novel. However, this discrepancy may be due to biases they identified in faecal lysis and DNA extraction methods they used, in addition to primer biases (Gill *et al.*, 2006). It is likely that, as more completed bacterial genomes and environmental metagenomes are submitted to the databases, the proportion of data showing no homology will reduce.

4.4.4 Protein Homologies

The potential functions encoded by the end-sequences were determined by Blastx analysis (Altschul *et al.*, 1990). Within each library genes encoding functions of

information storage and processing; cellular processes and signalling; metabolism; and gene transfer were found.

Of particular interest to this study is the homology of six clones to TnpV from *C. coli*. Further sequencing would determine if the *tnpV* gene is contained on a mobile element and if this element has spread to other species in the faecal metagenome. The *tnpV* gene resides on the integrative mobile elements Tn*4451*/Tn*4453* which harbour the *catP* gene for chloramphenicol resistance (Lyras *et al.*, 2004), it has also been associated with *tet*(O) (Scott, 2002) thus by sequencing out into the flanking DNA it can be determined which species in the faecal microbiota potentially harbour these resistance determinants.

The end-sequencing approach has not yielded complete genes, therefore the relationships illustrated in the cladogram may not be representative of the entire protein. All of the BAC TnpV sequences are distantly related to those in the databases suggesting that, if originally transferred as a functional module from a mobile element, this happened many years ago and the sequences have since diverged. Further work on these clones should include further sequencing into the insert to determine the full sequence of each *tnpV*. The phylogenetic relationship between the published *tnpV* sequences and the BAC *tnpV* sequences should then be repeated. Sequencing of the insert would also establish if these genes are contained on a mobile element and also if they are linked to an antibiotic resistance determinant.

One limitation of analysis of sequences from a metagenome is the large number that show homology to 'hypothetical' proteins. Without further investigation, no

supplementary information can be gained on the function of these sequences; however, they are useful in this study as they offer an idea of diversity. The number of such sequences is likely to decrease in the future due to expanding databases. Furthermore, functional screens of libraries, such as that performed in Chapter 5, are likely to contribute to this reduction by assigning functions to hypothetical genes.

4.4.5 Limitations

This study uses closest matches to determine if the libraries represent the total microbiota. If a description of species present is all that is required there are more efficient and accurate methods (Table 1.11) such as the use of 16S rRNA libraries. Metagenomic libraries have been used in this study because further analysis of the antibiotic resistance gene of each microbiota is desired (Chapters 5 and 6).

4.5 Conclusion

The current study has optimised the methods used to construct metagenomic libraries from oral and faecal samples; it has demonstrated that it is possible to construct metagenomic libraries of samples from these two environments containing DNA from representatives of all the genera commonly found in the microbiota. These libraries can then be exploited to gain information on both the phylogeny of the microbiota and also through functional screening to uncover rare or novel metabolic and biochemical processes such as antibiotic resistance.

CHAPTER FIVE

Investigating the Tetracycline and Erythromycin Resistance Determinants in the Oral and Faecal Microbiota of Six European Countries.

5.1 Introduction:

Culture independent techniques have allowed the presence of antibiotic resistance genes in different environments to be monitored using arrays (Frye *et al.*, 2006) and functional screens followed by the PCR of resistant clones to identify resistance determinants (Handelsman, 2004). Diaz-Torres *et al.*, (2006) recently described the oral tetracycline 'resistome' using the latter method, and found a predominance of *tet*(M), *tet*(O) and *tet*(Q) in healthy adults. The use of libraries also allows access to previously unsequenced DNA, and provides evidence of the *in situ* activities of bacteria within the microbiota (Handelsman, 2004; Riesenfeld *et al.*, 2004; Beja *et al.*, 2000a).

In the current study a macroarray of known tetracycline and erythromycin resistance determinants and the integrase genes of some mobile elements is used to determine the relative abundance of each in the total DNA extracted from the faecal and oral microbiota of six European countries. The results are then compared to the incidence of the same genes in the cultivable portion of each microbiota, and the genes recovered by the cloning and screening of BAC metagenomic libraries constructed from the total DNA from each environment. This allows the identification of any discrepancies each method introduces in the recorded levels of each determinant compared to the results gained from the array of total DNA extracted. Furthermore, the screening of the BAC libraries allows the potential discovery of novel resistance genes. This strategy is gaining popularity as a method for uncovering novel biochemical pathways (and antibiotic resistance determinants) from microbial metagenomes (Riesenfeld *et al.*, 2004; Diaz-Torres *et al.*, 2004)

This part of the study also investigates the cloning and expression of *tet*(S) in *E. coli*, to determine if it is expressed in the BAC host.

5.2 Materials and Methods

All methods used in this part of the study are outlined in Chapter two.

5.3 Results:

5.3.1 Macroarray of Total DNA Extracted from Each Sample

The total DNA extracted from each sample set from each country was analysed using the macroarray to determine which genes were present and which were the most common *tet* and *erm* genes in the microbiota of each environment (tables 5.1 and 5.2).

Spot intensity for all of the saliva samples was low relative to those of the faecal samples (Figure 5.1). Faecal *tet* genes accounted for up to 6.9 % of the hybridisation intensity of the 16S rDNA control compared to oral *tet* genes in which the maximum intensity is 0.9 % of the control (for calculation see Chapter 2).

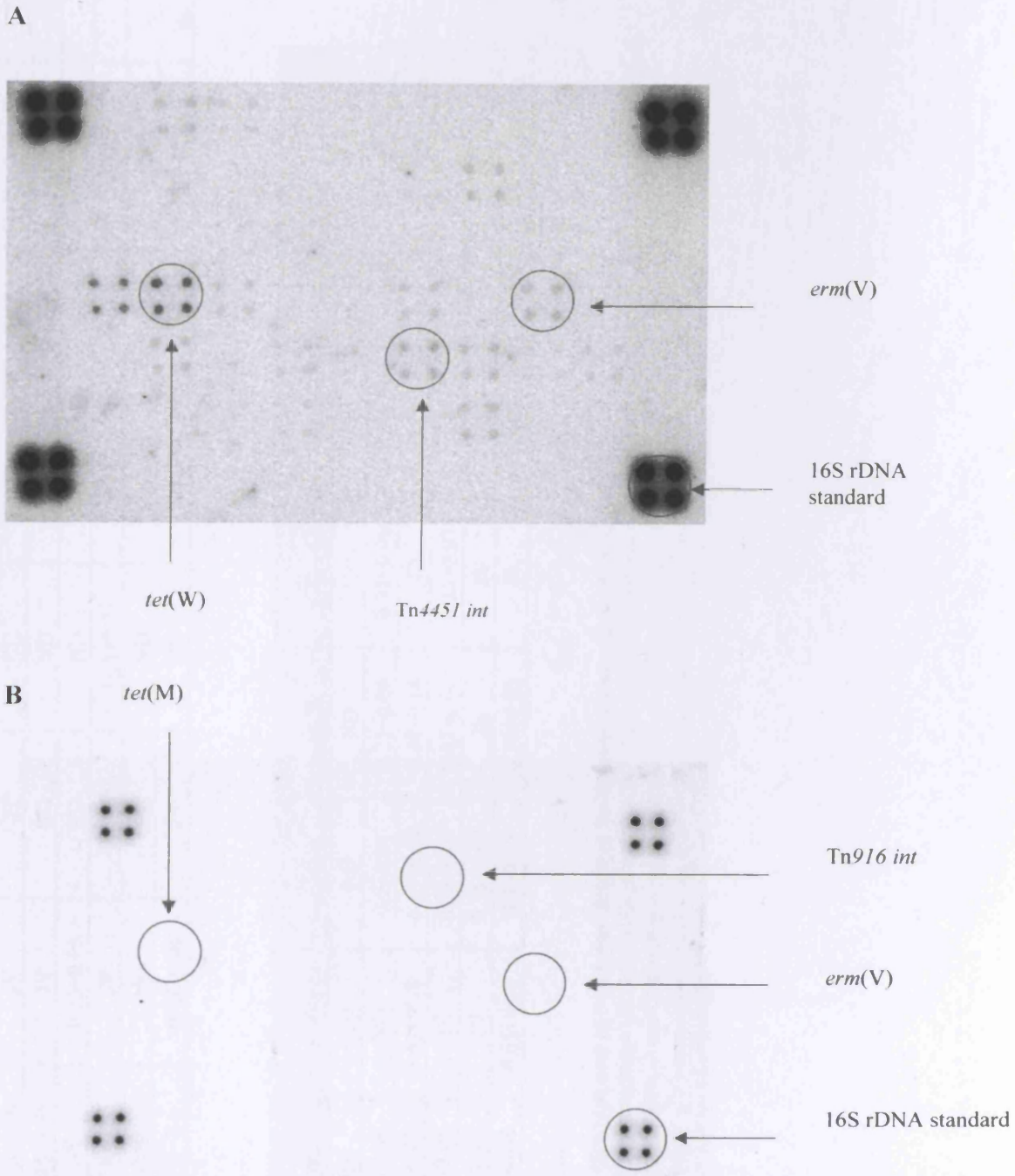


Figure 5.1: Comparison of the spot intensities for English faecal (A) and Scottish oral (b) total DNA hybridisations. Illustrating the different concentration so f bacterial DNA in each sample. N.B the English oral array is not shown since only *tet*(M) was detectable and comparisons between the different intensities is better illustrated with more genes. The macroarray layout is shown in Figure 2.3.

Hybridisation Intensity [a, b]	*tet*(M)	*tet*(Q)	*tet*(O)	*tet*(B)	*tet*(G)	*tet*(30)	*tet*(S)	JV[c]
England	0.13+0.06	JV	ND[d]	ND	ND	ND	ND	ND
Scotland	0.48+0.06	JV	ND	ND	0.2+0.04	0.1+0.04	0.15+0.03	W
Italy	JV	0.35+0.06	ND	ND	ND	ND	ND	W
Norway	0.38+0.05	JV	0.1+0.0	0.15+0.03	ND	0.1+0.04	ND	W
France	0.43+0.03	ND	ND	ND	ND	ND	ND	W
Finland	0.93+0.1	0.38+0.09	JV	ND	ND	JV	ND	W, D, Z, 32

A

Hybridisation intensity [a, b]	*erm*(B)	*erm*(F)	*erm*(V)	*erm*(E)	*erm*(C)	*erm*(G)	*erm*(X)	Tn*916 int*
England	ND	ND	ND	ND	ND	ND	ND	1.05+0.34
Scotland	0.45+0.09	ND	0.5+0.04	0.23+0.06	0.33+0.06	0.23+0.03	ND	0.63+0.05
Italy	0.43+0.05	1.63+0.28	0.53+0.08	0.48+0.14	ND	ND	0.7+0.06	0.33+0.06
Norway	0.38+0.05	JV	0.33+0.03	0.43+0.06	0.35+0.03	JV	ND	0.83+0.03
France	0.28+0.05	JV	0.25+0.03	JV	JV	ND	JV	0.83+0.05
Finland	0.58+0.08	0.65+0.03	0.4+0.06	0.18+0.05	JV	ND	JV	0.83+0.05

B

Table 5.1. Abundances of: a) *tet*; and b) *erm* genes detected by the macroarray in human saliva.

[a]Abundance is expressed as a percentage of the hybridisation intensity of the 16S rDNA control as determined by the AIDA Metrix alignment software.

[b]Intensities for each gene were are average of quadruplicate spots from which the standard error of the mean has been calculated.

[c]Genes that were 'Just Visible' (JV) were clearly visible by eye but intensities could not be calculated due to a high background.

[d] Genes that were not detected are labelled ND.

Hybridisation Intensity[a, b]	*tet*(W)	*tet*(O)	*tet*(Q)	*tet*(32)	*tet*(D)	*tet*(B)	*tet*(X)	*tet*(A)	*tet*(Z)	*tet*(30)
England	3.13±0.06	1.73±0.03	0.5±0.0	0.38±0.03	0.2±0.0	ND[d]	ND	0.13±0.03	0.15±0.05	JV[c]
Scotland	2.08±0.03	2.0±0.04	0.53±0.03	0.2±0.04	0.1±0.0	ND	ND	ND	ND	ND
Italy	6.9±0.85	2.93±0.39	1.93±0.4	JV	ND	ND	1.4±0.7	ND	ND	ND
Norway	2.08±0.3	1.93±0.06	0.35±0.03	0.28±0.03	ND	ND	ND	ND	JV	ND
France	3.05±0.06	2.25±0.06	1.28±0.03	0.58±0.03	0.1±0.0	0.2±0.0	2.13±0.05	JV	ND	ND
Finland	3.2±0.04	3.3±0.07	0.6±0.0	0.53±0.03	ND	ND	ND	ND	ND	ND

A

Hybridisation Intensity[a,b]	*erm*(B)	*erm*(F)	*erm*(G)	*erm*(V)	*erm*(E)	*erm*(X)	Recombinase of Tn*4451*	Integrase of Tn*1549*
England	0.4±0.0	0.35±0.03	ND	0.58±0.08	0.43±0.05	ND	0.83±0.03	0.2±0.0
Scotland	0.25±0.03	0.3±0.03	0.5±0.0	0.1±0.0	JV	ND	0.3±0.0	JV
Italy	2.93±0.72	JV	ND	JV	JV	ND	JV	ND
Norway	0.65±0.05	0.1±0.0	0.3±0.04	0.1±0.0	JV	ND	0.2±0.0	JV
France	2.9±0.05	1.55±0.09	0.38±0.03	0.28±0.03	0.28±0.03	0.1+0.0	0.35±0.03	0.08±0.03
Finland	0.63±0.03	0.55±0.03	ND	0.15±0.03	0.1±0.04	ND	0.18±0.03	0.28±0.03

B

Table 5.2. Abundances of: a) *tet*; and b) *erm* genes detected by the macroarray in human faeces.

[a]Abundance is expressed as a percentage of the hybridisation intensity of the 16S rDNA control as determined by the AIDA Metrix alignment software.

[b]Relative intensities for each gene were are average of quadruplicate spots from which the standard error of the mean has been calculated.

[c]Genes that were 'Just Visible' (JV) were clearly visible by eye but intensities could not be calculated due to a high background

[d] Genes that were not detected are labeled ND

5.3.1.1 Total Oral DNA Hybridisation

5.3.1.1.1 Tetracycline Resistance

tet(M) was the most commonly isolated tetracycline resistance determinant in all oral samples except that from Italy. Spot intensity as a percentage of the 16S hybridisation ranged from 0.13 % in the English sample to 0.93 % in the Finnish sample.

tet(Q) was the most commonly identified tetracycline resistance gene in the Italian sample (occurring at 0.35 % of the 16S spot intensity, compared to *tet*(M) which was just visible). It was present in all other samples except that from France, however, spot intensities were low relative to *tet*(M), in most cases they were just visible.

All samples except the English harboured *tet*(W). Other tetracycline resistance genes present in more than one sample were *tet*(30) (Scottish, Norwegian and Finnish samples); and *tet*(O) (Norwegian and Finnish samples).

5.3.1.1.2 Erythromycin Resistance

erm genes were detected in all samples except that from England. *erm*(B), *erm*(E) and *erm*(V) were present in all other samples.

erm(V) was the most common gene in the Scottish and Italian samples occurring at 0.5 % and 0.53 % of the relevant 16S spot intensities respectively.

erm(B) was the predominant *erm* gene in the French sample (0.28 % of the 16S hybridisation); *erm*(E) was the most common *erm* gene in the Norwegian sample

(0.43 % of the 16S hybridisation), and *erm*(F) the most common in the Finnish sample at 0.65 % of the 16S spot intensity for that sample.

5.3.1.1.3 Integrase Genes

All oral samples contained the integrase gene, *int*, from Tn*916*. It was most abundant in the English sample (1.05 % of the 16S intensity), and least abundant in the Italian sample (0.33 %).

5.3.1.2 Total Faecal DNA hybridisation

5.3.1.2.1 Tetracycline Resistance

tet(W) was the most abundant gene in all samples except the Finnish in which in was *tet*(O), at 3.3 % of spot intensity of the 16S control, compared to 3.2 % for the *tet*(W) probe.

The Italian sample has the greatest percentage hybridisation to the tetracycline gene (*tet*(W)) compared to the 16S rRNA control (spot intensity at 6.9 % of its 16S).

tet(O) featured in all sample sets as the second most common tetracycline resistance determinant (except for Finland, see above). Percentages of 16S hybridisation intensity ranged from 1.73 % (England) - 2.93 % (Italy).

tet(Q) was the third most common gene in all faecal samples (with the exception of the French sample in which *tet*(X) is the third most common at 2.13 % of 16S spot intensity).

tet(32) occurred in all samples. However, it should be noted that the *tet*(32) probe does not distinguish between complete *tet*(32) genes and *tet*(O)/*tet*(32)/*tet*(O) hybrids.

No *tet*(M) was found in the faecal DNA, problems with the array were ruled out since the arrays are printed in sets of 16 by the Biorobotics Microgrid II System and the same array set was used for the saliva and faecal DNA hybridisations.

5.3.1.2.2 Erythromycin Resistance

The most common *erm* gene differed from sample to sample. *erm*(B) was the most common in the French (2.9 % of the 16S hybridisation), Finnish (0.63 %), Italian (2.93 %) and Norwegian (0.65 %) samples.

erm(V) was the most common erm gene in the English sample occurring at a spot intensity of 0.58 % of the 16S hybridisation.

erm(G) was the most common *erm* gene in the Scottish sample (0.5 % of the 16S intensity), but only occurred in two other samples (French and Norwegian).

5.3.1.2.3 Integrase / Recombinase Genes

Of the transposons probed for the recombinase of Tn*4451* was detected in all faecal samples, and the integrase of Tn*1549* was detected in all of the samples except that from Italy.

The DNA did not hybridise to the probe for *int* from Tn*916*.

5.3.2 Metagenomic BAC Libraries

Twelve metagenomic BAC libraries have been constructed (Table 5.3). Each has an average insert size in the range of 21 – 35 Kb, and each represents over 44 Gb of DNA from its corresponding microbiota. The English oral and faecal libraries are those previously reported in Chapter 4.

Sample Origin	Library	Mean Insert Size (Kb)	No. of Clones	Equivalent no. bacterial genomes	Total Gb
England					
	Saliva	30	1.50×10^6	9967.1	44.85
	Faecal	24	1.99×10^6	10 592.4	47.67
Finland					
	Saliva	21	2.34×10^6	10 941.5	49.24
	Faecal	32	1.51×10^6	10 708.8	48.19
France					
	Saliva	30	1.54×10^6	10 243.0	46.09
	Faecal	30	2.10×10^6	13 967.2	62.85
Italy					
	Saliva	27	1.82×10^6	10 914.0	49.11
	Faecal	30	1.73×10^6	11 514.0	51.82
Norway					
	Saliva	28	1.65×10^6	10 272.0	46.22
	Faecal	28	1.62×10^6	10 100.0	45.45
Scotland					
	Saliva	35	1.35×10^6	10 476.0	47.14
	Faecal	25	1.78×10^6	9885.0	44.48

Table 5.3: Metagenomic BAC libraries. Equivalent number of bacterial genomes is based on an average genome size of 4.5 Mb (the average genome size in the databases at the time of calculations).

5.3.2.1 Screening of the BAC Libraries

The libraries were screened for inserts conferring tetracycline resistance. 1100 Mb (English), 822 Mb (Finnish), 113 Mb (French), 65 Mb (Norwegian), and 1320 Mb (Scottish) of the oral libraries were plated onto selective media.

102 Mb (English), 124 Mb (Finnish), 754 Mb (Italian), 1260 Mb (Norwegian) and 53 Mb (Scottish) of the faecal libraries were also screened in a similar way.

32 tetracycline resistant clones were found when using a concentration of 10 μg/ml tetracycline in the selective media. Twenty three were from oral libraries (7 from the English library; 6 from the Finnish library; 2 from from French library; and 8 from the Scottish library); 9 were from faecal libraries (1 from the English library; 4 from the Italian library; 3 from the Norwegian library; and 1 from the Scottish library). However, when re-grown from glycerol stocks, only four were found to grow on tetracycline.

5.3.2.2 Analysis of Tetracycline Resistant Clones

Tetracycline resistant clones were analysed on the array. They were probed with *tet*(M), *tet*(O), *tet*(W), *tet*(Q), *tet*(32) and probes corresponding to the efflux and enzymatic *tet* genes in one hybridisation (*tet*(A), *tet*(B), *tet*(C), *tet*(D), *tet*(E), *tet*(G), *tet*(H), *tet*(J), *tet*A(P), *tet*(Y), *tet*(Z), tet(X) and *tet*(30) but not *tet*(K), *tet*(L), *tet*(V), *tet*(31), *tet*(33), *tet*(35), *tet*(38), *tet*(39), *tcr*3, *otr*(B) and *otr*(C) which were not included in the probes), and in a further hybridisation the remaining RPP *tet* genes (*tet*B(P), *tet*(S), *tet*(T), *tet*(W), *tet*(32) and *tet*(36) but not *tet* and *otr*(A)).

One clone from the Scottish faecal library, assigned SFtetC10 hybridised to the *tet*(M) probe, but not the Tn*916* integrase probe.

One clone, NFtetC1, from the Norwegian faecal library hybridised to the *tet*(O) probe.

The two other clones, IStetC2 (from the Italian oral library) and FRStetC11 (from the French oral library) failed to hybridise to any probes on the array.

5.3.2.2.1 SFtetC10 (*tet*(M) Clone)

A PCR reaction targeting the sequence immediately flanking the *tet*(M) gene in Tn*916* was performed on the *tet*(M) containing clone, SFtetC10 using primers RT1 and RT2 and RT3 and RT4 (Figure 5.2). Both were positive (data not shown). A PCR reaction targeting the integrase, *int*, gene Tn*916* on the SFtetC10 clone gave a negative result. End-sequencing (see Figure 2.1) determined the insert showed no significant homology to anything in the databases (Appendix 9).

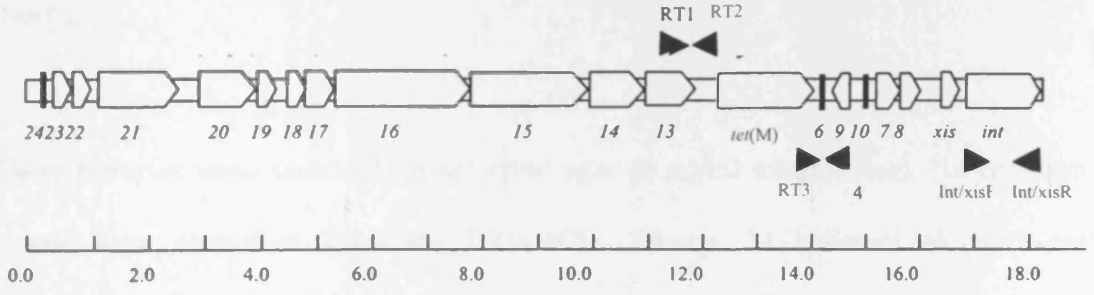


Figure 5.2: Schematic of Tn*916* and location of the primers used inPCR reactions. Small black arrows indicate the primers used to check the flanking sequence of the *tet*(M) gene (Tn916 schematic taken from Flannagan & Clewell, 2004).

5.3.2.2.2 NFtetC1 (*tet*(O) Clone)

The *tet*(O) gene of the NFtetC1 clone was sequenced (Appendix 10) and found to be closely related to the *tet*(O) from *Streptococcus pneumoniae*. Its support is characterised in Chapter six.

5.3.2.2.3 IStetC2 and FRStetC11 (Unknown Determinants)

The tetracycline resistant clones, IStetC2 (which contained an insert of 90.2 Kb) and FRStetC11 containing unknown tetracycline resistance determinants were subcloned into pUC19 as described in section 2.10 (Table 5.4).

	FRStetC11	IStetC2
Average Insert Size	6.8	7.4
Mb Screened	22.37	17.91

Table 5.4: pUC19 subclone libraries of the tetracycline resistant BAC clones FRStetC11 and IStetC2

These libraries were screened on selective agar (8 μg/ml tetracycline). No resistant clones were identified from the FRStetC11 library. 14 resistant clones were identified from the IStetC2 library. One sub-clone with an insert of 8.5 Kb was identified for end-sequencing. 1494 bp sequence was gained from sequencing using the M13F primer and subsequent sequencing reactions (Appendix 11). This sequence had 99 % homology at the DNA sequence level to the pRSB107 plasmid from an uncultured bacterium, (accession number AJ851089) bp 55597-57091 encoding TnpA, YbjA (hypothetical protein) and CatA (Figure 5.3). It also showed 99 %

homology to pR100 from *E. coli* (Accession number AP000342.1) and 44 other sequences from Gram negative organisms, including a multidrug resistance locus in *Acinetobacter baumannii* (accession number CT025832.1), a pathogenicity island in *Shigella flexneri* (accession number AF326777.3), and plasmids from *Klebsiella pneumoniae* (pRMH760, accession number AY123253.3), *Serratia marcescens* (pR478, accession number BX664015.1), and *E. coli* (pNRI, accession number DQ364638.1).

The M13R primer and subsequent sequencing reactions into the insert produced 2582 bp sequence (Appendix 11) which had 98 % homology at the nucleotide level to the plasmids R64 from *S. typhimurium* (Accession number AP005147). BlastX searches identified the region of R64 cloned (bp 35612-38194) as encoding the hypothetical protein YdfB and Mck which itself shows homology to VagD from the *S. dublin* virulence plasmid (Figure 5.3).

All the above plasmids contain the tetracycline resistance determinants *tet*(A). In addition, pRSB107 and pR100 also harbour *tet*(C), *tet*(D) and *tet*(R).

The sub-clone was not fully sequenced due to financial and time constraints.

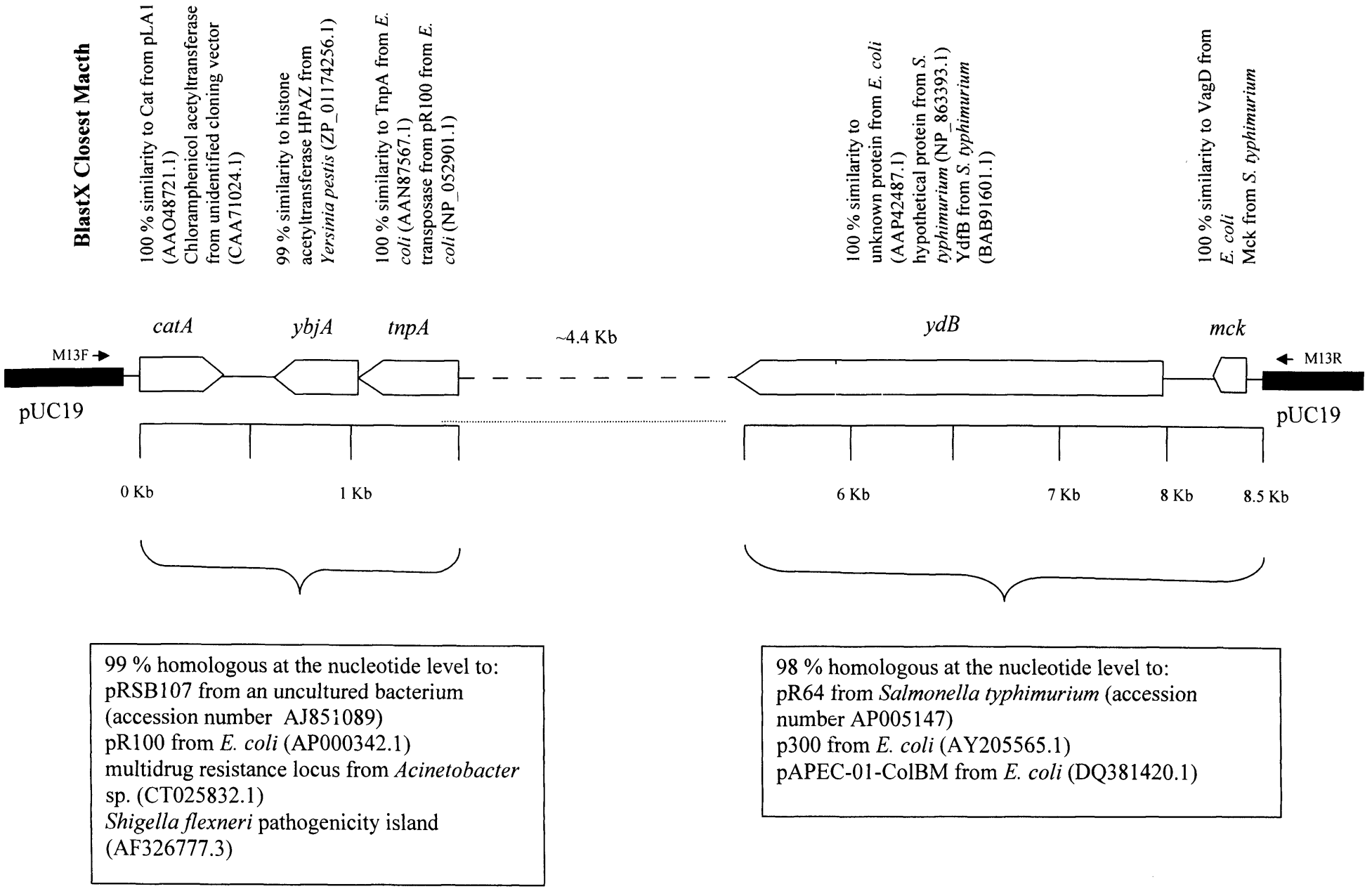


Figure 5.3: Schematic of the Blastn and Blastx homology searches for end-sequences of the sub-clone of IStetC2.
Open arrows indicate genes from the first listed blastn match. Small black arrows indicate primers used. Insert produced using *Hin*dII digestion

5.3.3 Metagenomic pDL278 Libraries

Since it is known that only ~60 % of genes from Gram positive organisms express in a *E. coli* (Rondon *et al.*, 1999), the vector pDL278 (Figure 2.2) was employed to construct small insert metagenomic libraries. This vector has been proven to generate random, representative single species gene banks, and is capable of stable maintenance in *E. coli, E. faecalis* and various species of streptococci (Dunny *et al.*, 2001). The vector has a polylinker containing 19 restriction enzyme recognition sites, a spectinomycin resistance marker gene and fragments of DNA up to 10 Kb can be cloned (figure 2.2) (Dunny *et al.*, 1991).

Metagenomic libraries of the English oral and faecal samples were constructed in pDL278. The inserts were sized in *E. coli* hosts, the average insert size for both libraries was ~8 Kb. The oral library contained 48.4 Mb of insert DNA; the faecal library 42.3 Mb. The vector-insert ligation product was also transformed in to *S. mutans*, the inserts sizes were also ~8 Kb, and ~25 Mb of each was screened on 8 μg/ml tetracycline. No tetracycline resistant clones were found.

5.3.4 Expression of *tet*(S)

As discussed above, not all genes express in a heterologous host. Indeed, Gabor *et al.*, (2004) estimate that only ~40 % of genes recovered by random cloning of a microbiota can be expected to express in *E. coli*. In order to investigate if *tet* genes expressed when cloned in *E. coli* a single species BAC library was made using genomic DNA extracted from *E. faecium* 664:1H1 known to contain the *tet*(S) gene.

The average insert size of the library was 29.8 Kb, 38.6 Mb were screened on 10 μg/ml tetracycline, no tetracycline resistant clones were found.

The library was plated out (74.4 Mb) and colony blotting experiments were performed using a probe derived from a PCR reaction of the internal region of the *tet*(S) gene. One colony that hybridised strongly to this probe was shown to contain the *tet*(S) gene. The gene was sequenced and found to be identical to the *tet*(S) from *Listeria monocytogenes* (accession number L09756.1)); 99 % similar to *tet*(S) from *L. lactis* plasmid pK214 (accession number X92946); and 98 % similar to *tet*(S) from Tn*916*S (accession number AY534326). However it did not confer tetracycline resistance in *E. coli* (A Roberts *et al.*,, 2006).

5.4 Discussion:

5.4.1 Resistance in the Oral Microbial Metagenome

5.4.1.1 Tetracycline resistance

5.4.1.1.1 *tet*(M)

The results of the macroarray hybridisation of the total metagenomic DNA illustrate that *tet*(M) is the most common tetracycline resistance determinant in the oral cavity. This agrees with the study on the cultivable flora (Chapter 3), and previous studies on the distribution of *tet* genes in the oral cultivable flora (Villedieu *et al.*, 2003, Lancaster *et al.*, 2003; Lacroix & Walker, 1995; Olsvik *et al.*, 1994), and the oral microbial metagenome (Diaz-Torres *et al.*, 2006). *tet*(M) is usually found on the Tn*916* family of conjugative transposons (Roberts *et al.*, 2001a,b). The Tn*916* integrase was found in all the oral metagenomes, suggesting that this is one of the vehicles of *tet*(M) dissemination in the oral cavity. Furthermore, Tn*916* is commonly found in oral bacteria (Mercer *et al.*, 2001; Bentorcha *et al.*, 1992).

5.4.1.1.2 *tet*(Q)

tet(Q) was the second most commonly detected *tet* gene in contrast to the results of the culture-based study which identified *tet*(O) as the second. Furthermore, the metagenomic study performed by Diaz-Torres *et al.* (2006) also identified *tet*(O) as the second most common resistance determinant, although *tet*(Q) the third most common. *tet*(Q) has been previously reported in various oral genera including *Bacteroides* spp., *Porphyromonas* spp. and *Prevotella* spp. (Chung *et al.*, 2002; Sanai *et al.*, 2002), which are anaerobic, this could go some way to explaining the discrepancy between the incidence of *tet*(Q) in the total DNA and that found in the cultivable flora. The difference between this study and the Diaz-Torres study can be

explained by sampling and random variation since the number of *tet*(O) genes found in their study was only one more than the *tet*(Q) genes found.

5.4.1.1.3 *tet*(O)

tet(O) was only present in the Norwegian and Finnish metagenomes at very low levels. It was absent in all other oral samples. In contrast, in previous studies it was the second most commonly isolated *tet* gene using a metagenomic approach (Diaz-Torres *et al.*, 2006). As with all genes on the array, the incidence of *tet*(O) in the oral samples may appear reduced compared to previous studies as the total DNA extracted includes human DNA at a high concentration (61.0 %) (Chapter 4), which would dilute the bacterial DNA and thus lower the hybridization signal. This could also explain why the hybridisation intensities of the oral samples are low in comparison to the faecal samples (The sample of the English faecal library examined contained no detectable human DNA). This 'dilution effect' would mask genes present at low concentrations in the oral metagenomic DNA.

5.4.1.2 Erythromycin Resistance

No *erm* genes were detected in the English oral sample. This is most likely due to the high incidence of human DNA in the sample masking the presence of genes at low concentrations. *erm* genes were found in other samples, therefore a similar end-sequence analysis would need to be performed to determine if the amount of human DNA present in other samples was lower than that in the English sample. If not, then the incidence of *erm* genes in the English oral sample is much lower than that for other countries.

The most common *erm* gene differed between samples.

erm(V) was the most common gene in the Scottish and Italian samples in contrast to the culture study in which it did not feature among the most common genes. *erm*(V) has been previously reported in oral species (reviewed in Roberts *et al.*, 1999b), but mobile genetic elements have yet to be characterised that harbour this gene.

erm(B), *erm*(E) and *erm*(F) are the most common erm genes in the French, Norwegian and Finnish samples, respectively. In the culture study *erm*(B) was the most common, followed by *erm*(F) and *erm*(E). Thus one determinant does not predominate, and no obvious difference can be found between the aerobic cultivable and total oral microbiota. The presence of *erm*(F) in the common *erm* genes may be in part explained by the fact that it has commonly been found in the same oral isolate as *tet*(Q) (Chung *et al.*, 2002) which has been identified as the second most common tetracycline resistant gene. *erm*(F) and *tet*(Q) have been found together in up to 48% of oral *B. forsythus*, *P. ginigivalis* and *P. intermedia* isolates, however, any genetic support that links the two has yet to be characterised (Chung *et al.*, 2002).

5.4.2 Resistance in the Faecal Microbial Metagenome

5.4.2.1 Tetracycline Resistance

5.4.2.1.1 *tet*(W)

In the faecal metagenome, *tet*(W) was the most common *tet* gene, occurring at between 2.08 % (Scotland) – 6.90 % (Italy) of the 16S rDNA hybridisation intensity. *tet*(W) was only found in 2.6 % of the culture isolates. Previous studies have found *tet*(W) is common among anaerobic isolates (Masco *et al.*, 2006; Kastner *et al.*,

2006; Stanton *et al.*, 2004) which the current culture study (Chapter 3) did not isolate.

5.4.2.1.2 *tet*(O)

tet(O) is the second most common *tet* gene in the total faecal DNA and in the culture study. It is harboured by both aerobic and anaerobic species. As with the culture study, its wide-spread presence is likely to be due to it being contained on mobile genetic elements. Only two of these have been characterised, pTet and pCC31 from *C. jejuni*, which transfer *tet*(O) between *Campylobacter jejuni* strains (Avrain *et al.*, 2004). However, *tet*(O) has also been linked to *mef*(A) in Streptococci: in the *S. pyogenes* genome a single 260 kb band hybridised with both the *tet*(O)- and the *mef*(A)-specific probe in Southern blot analysis (Giovanetti *et al.*, 2003), suggesting the presence of *tet*(O) on an integrative mobile element.

5.4.2.1.3 Other *tet* Genes

tet(Q) and *tet*(32) were also common in the metagenomic DNA. They are less common among the cultivated isolates suggesting they are more wide-spread among the uncultivated / obligately anaerobic species. Evidence supporting this theory includes the fact that *tet*(Q) is wide-spread among anaerobic *Bacteroides* spp., (up to 80 % of isolates have been found to harbour it (Shoemaker *et al.*, 2001)) which are present in large numbers in the human colon (Lepine *et al.*, 1993), and *tet*(32) has only been found in relatively few species, one of which is the *Clostridium*-related human colonic anaerobe K10 (Melville *et al.*, 2001). Furthermore, sequence analysis of Bacteroides *tet*(Q) genes implicates horizontal gene transfer in both its inter- and

inta-generic spread (Nikolich *et al.*, 1994a). Virtually identical *tet*(Q) sequences were found in *B. fragalis* and *B. thethaiotaomicron* (99 % identity) and in *B. fragalis* and the distantly related *Prevotella ruminocola*. Furthermore, Shoemaker *et al.*, (2001) demonstrated the *tet*(Q) gene from 10 different *Bacteroides* spp. and the *tet*(Q) from the conjugative transposon CTnDOT showed between 96-100 % identity, implicating the element in its spread.

tet(M) is below the detection level of the array for the faecal metagenomic samples, but is common in the cultivables. The same is true for the Tn*916* integrase, suggesting that these genes are more common among the cultivable aerobic / facultative anaerobic species of the faecal microbiota which are out numbered 300:1 by the obligate anaerobes (Blaut *et al.*, 2002). Furthermore, the faecal microbiota is thought to be composed of up to 80 % uncultivable species (Duncan *et al.*, 1993; Hayashi *et al.*, 2002; Suau *et al.*, 1999) therefore, the high incidence of *tet*(M) and Tn*916* in the cultivable flora does not mean that it is as common in the total microbiota.

Interestingly, *tet*(A) and *tet*(B) have been found in up to 51 % of tetracycline resistant faecal *E. coli* strains (Karami *et al.*, 2006), but are only detected at low levels in the English and French libraries, respectively. Although it is estimated that *E. coli* only make up 0.1 % of the total microbiota (Blaut *et al.*, 2002), *tet*(A) resides on the plasmids pRAS1 and pIE420 (Rhodes *et al.*, 2000) and the transposon Tn*1721* (Allmeier *et al.*, 1992), all of which have been shown to spread to *Salmonella* sp., a common inhabitant of the colon (Upreti *et al.*, 2004). *tet*(B) resides on Tn*10* (Kimura *et al.*, 1998). Therefore the potential for these genes to spread in the faecal microbiota is evident.

5.4.2.2 Erythromycin Resistance

As with the oral DNA, *erm* genes are present at low concentrations compared to *tet* genes and the most common *erm* gene differed from country to country.

erm(B) is commonly detected in all samples. *erm*(B) is a common macrolide resistance determinant among eneterococci and streptococci which both inhabit the human colon (Min *et al.*, 2003). As with the cultivable isolates, the high incidence of *erm*(B) is probably due to its presence on mobile genetic elements, including Tn*5398*, a mobilisable non-conjugative element (Farrow *et al.*, 2001), and Tn*916*-like elements (Giovanetti *et al.*, 2003). Furthermore, the in vitro transfer of *erm*(B) from *C. difficile* to *B. fibrisolvens* (both common components of the faecal microbiota (Blaut *et al.*, 2002) has been demonstrated (Spigaglia *et al.*, 2005a), suggesting it might also occur in the gut. Also common in the metagenome is *erm*(F) which is also maintained on mobile genetic elements (Table 1.6). *erm*(F) is harboured by the *Bacteroides* spp. plasmids pBF4 (Shoemaker *et al.*, 1985), pBI136 (Smith & Macrina, 1984) and pBFTM10 (Tally *et al.*, 1982) and the conjugative transposon CTnDOT (Whittle *et al.*, 2001) which are all capable of intra-generic transfer. Since *Bacteroides* spp. are major components of the faecal microbiota (Blaut *et al.*, 2002; Upreti *et al.*, 2004) it is possible that the spread of these elements is responsible for the high incidence of *erm*(F).

5.4.2.3 Integrase / Recombinase Genes

The recombinase gene of Tn*4451* was commonly found in the faecal microbiota of all countries, and the integrase of Tn*1549* in all but the Italian sample. Tn*4451*

harbours a chloramphenicol resistance genes and is found among *Clostridium* spp. (Abraham & Rood, 1987; Crellin & Rood, 1998), which goes towards explaining its high incidence in the faecal samples since Clostridia are amongst the most common species in this environment (Mueller *et al.*, 2006).

Tn*1549* was first isolated from an enterococcal isolate (Garnier *et al.*, 2000). It is a 34 Kb transposon, organised similarly to the Tn*916* family of CTns, however, the *vanB2* operon replaces the *tet*(M) gene in the central region and the conjugation region shows differences (Figure 1.10) (Burrus *et al.*, 2002). It is surprising that the Tn*1549* integrase is so common considering that no Gram positive isolates were found to be vancomycin resistant (Chapter 3). However, since *tet*(W) is harboured on the Tn*B1230* conjugative transposon which encodes transfer proteins with up to 67 % identity to some encoded by Tn*1549* (Melville *et al.*, 2004) it is possible that the probe cross hybridised to the *tet*(W) support, especially since *tet*(W) was the most common resistance determinant found in the faecal microbiota. Also it is possible that there is a common genetic element present in this metagenome which is as yet uncharacterised as it may not contain a readily selectable marker. Further sequence information of the two integrase genes would be needed before this can be concluded.

5.4.3 Metagenomic Libraries

5.4.3.1 Clone Stability

Having initially found 32 tetracycline resistant BAC clones, only four grew on tetracycline following storage at –70°C in glycerol stocks. It is possible that some of these clones contained mobile elements which are highly unstable and were lost over the period of storage (approximately 10 weeks). This has been reported before for

Tn*916* (Garwon-Burke *et al.*, 1984), and for Tn*4451* (Abraham & Rood, 1987). Since all the clones retained chloramphenicol resistance, the BAC vector must still be contained in the *E. coli* host. It is also possible that BACs do not always stably maintain inserts despite the presence of the *par* operon (Easter *et al.*, 1998). Furthermore, various groups have reported a similar phenomenon (Karen Scott, personal communication 2005; Arnfinn Sundsfjord, personal communication, 2005). In future work this problem could be overcome by immediate sub-cloning to isolate resistance genes in the absence of the complete mobile element, and immediate plasmid isolation.

In comparison to the Diaz-Torres study (2006) which screened ~27.78 Mb of insert DNA and found 58 tetracycline resistant clones (21 contained known resistant determinants), the current study screened 5713 Mb and found only 32 tetracycline resistant clones. This difference may be explained by the use of different vectors and their associated ability to maintain inserts stably, discussed above. Furthermore the copy number of BAC is one-two per cell (Shizuya *et al.*, 1992) compared to ~700 for pUC19. This may influence expression since cloned DNA in pUC will have a higher gene dosage, i.e. more Tet protein would be produced which would exert more of an effect on the host cell. In addition, due to the small insert size in pUC clones, the *tet* genes would be in close proximity to the strong pUC promoters, compared to HMW BAC inserts. If the promoter on a BAC insert does not function in the heterologous host and the *tet* gene is located far from the BAC promoter, the gene may not express (Rondon *et al.*, 1999; Gabor *et al.*, 2004).

5.4.3.2 Expression of BAC Insert DNA in *E. coli*

The *tet*(S) gene from *E. faecium* EfcTn1 does not express in *E. coli*. Previously it was widely thought that genes from a Gram positive host would express in *E. coli*, however, this proves that not to be the case. More studies of this kind are required to establish which tetracycline resistance genes express in *E. coli*. Various shuttle vectors are now used in order to screen metagenomic libraries in a variety of hosts. These studies have found different expression patterns depending on the host (Martinez *et al.*, 2004). pDL278 is a shuttle vector capable of maintenance and replication in a variety of Gram negative and Gram positive hosts. English oral and faecal libraries were constructed in this vector and transformed into *S. mutans* even though ~25 Mb of each library was screened no tetracycline resistant clones were found. However, this is not surprising since the incidence of resistant clones in the BAC library is one per 178.5 Mb, and the insert size in the pDL278 library is ~10 kb (cf. 25-30 Kb in BACs). Further screening of these libraries would allow a comparison to be made between the determinants expressed in a Gram positive host and those expressed in *E. coli*.

5.4.3.3 Tetracycline Resistant Clones

5.4.3.3.1 SFtetC10 (*tet*(M) Clone)

End-sequencing of the Scottish faecal clone that hybridised to the *tet*(M) array probe revealed the insert to be novel and likely from a previously unsequenced strain, possibly an uncultured bacterium (end-sequences showed no homology to anything in the databases), further extending the support range of the *tet*(M) determinant. PCR analysis revealed the sequences immediately flanking *tet*(M) are from Tn*916*, suggesting the *tet*(M) is contained on at least a fragment of this element. However,

the clone did not hybridise to the Tn*916 int* probe. *tet*(M) is maintained on numerous mobile elements, some of which are composite elements (Table 1.6), including Tn*5397* which differs from Tn*916* in that it has a different integration/excision module and contains a group II intron (Roberts *et al.*, 2001a). The evolution of mobile elements in discussed in Chapter 1 where it is explained that they can exchange functional modules including integrase genes. It is possible that SFtetC10 harbours a Tn*5397* derivative or another unknown element. Alternatively, Tn*916* could have been responsible for the transfer of *tet*(M) to the host strain and the integrase subsequently lost. Sub-cloning and sequencing of the insert would allow us to determine the genetic support of this gene. The sequence of which could then be used to design novel probes for the array so that it's incidence in the faecal metagenome could be determined.

5.4.3.3.2 NFtetC1 (*tet*(O) Clone)

The other tetracycline resistant clone that hybridised to an array probe was found to contain an insert from a *Clostridium* sp. and to harbour a *tet*(O) gene showing 98 % identity to the *tet*(O) from *Streptococcus pneumoniae*. *tet*(O) has been found on the *C. jejuni* plasmids pTet and pCC31, and it has been found to transfer rapidly and without antibiotic selection between *C. jejuni* strains in the laboratory (Avrain *et al.*, 2004). It has also been commonly found in Streptococci where it has been linked to either *mef*(A) or *erm*(A) (Giovanetti *et al.*, 2003). To obtain information about the *tet*(O) support primers were designed in order to sequence out from the *tet*(O) gene. The results of these experiments are discussed in Chapter six.

5.4.3.3.3 IStetC2 and FRStetC11 (Unknown Determinants)

Two clones were found to be tetracycline resistant in functional screens, but failed to hybridise to any probes on the array. The array does not contain probes for all tetracycline resistance determinants, so the genes conferring resistance could either be rare (*tet*(K), *tet*(L), *tet*(V), *tet*(31), *tet*(33), *tet*(35), *tet*(38), *tet*(39), *tet*, *tcr*3, *otr*(A), *otr*(B) and *otr*(C)) or previously unreported.

End sequencing analysis has found the IStetC2 clone is likely to originate from a *Salmonella* sp.. IStetC2 had an insert of 90.2 Kb, and was sub-cloned into pUC19. Sequencing reactions into the insert were performed on one tetracycline resistant subclone using M13 primers. The insert of 8.5 KB was not fully sequenced due to time constraints.

The 5' end of the insert (1496 bp sequence) was found to have 99 % identity at nucleotide level to pRSB107 from an uncultured bacterium which contains *tet*(D), *tet*(C), *tet*(A) *tet*(R). It also shows 99 % homology to 44 other sequences from Gram negative organisms, including a multidrug resistance locus from *Acinetobacter* sp., a pathogenicity island from *Shigella* sp., and plasmids from *Klebsiella* sp., *Serratia* sp., and *Escherichia* sp., showing that this sequence is common among Gram negatives and potentially transfers as a functional module between mobile genetic elements.

The 3' end of the insert (2640 bp sequence) was found to have 99 % identity to the *S. typhimurium* plasmid R64 which also harbours *tet*(A), and the *E. coli* plasmids p300 and pAPEC-01-Col which do not harbour a tetracycline resistance gene. It is likely that the insert is a natural chimera which has evolved through the recombination and / or exchange of functional modules.

Therefore it is possible that IStetC2 contains *tet*(A) or one of the other *tet* genes harboured by pRSB107 (*tet*(C), *tet*(D) and *tet*(R)). If this is the case, the *tet* genes were probably missed by the array due to sequence divergence in the region probed. This could be confirmed by sequencing the whole insert. It illustrates the constraints of the array in that some genes may not be detected if their sequence differs from that of the probe. Therefore all clones that do not hybridise to probes on the array should be checked by PCR to determine their presence. The same problem has been encountered before when using arrays. Frye *et al.*, (2006) found that some *tet*(A) genes were not detected from control strains due to the fact that their sequence was divergent from that used to construct the probes on the array. This finding suggests that the levels of some genes, certainly *tet*(A), in the metagenomes investigated in this study may be higher than suggested by the array results.

However, probes for *tet*(A), *tet*(C), *tet*(D) and *tet*(R) are used in the array anaylsis of the clones. Therefore, another possibility is that the natural chimera cloned in IStetC2 harbours a novel tetracycline resistance gene. To establish the genetic basis for resistance, the entire insert of the subclone should be sequenced.

An alternative explanation for the apparent chimera is that it is a cloning artefact. Sequencing the complete insert and looking for an abrupt change at a *Hin*dIII site would allow its identification.

No tetracycline resistant sub-clones of the FRStetC11 BAC were found. Partial digestion of the original clone was performed in order to create the sub-clones suggesting the lack of resistant sub-clones is not due to choice of restriction enzyme. Further rounds of sub-cloning would need to be performed in order to isolate a

tetracycline resistant sub-clone for sequencing. This clone should be fully sequenced to determine which tetracycline resistance gene is present.

5.5 Conclusions

In this part of the study metagenomic analysis has established that culture studies do not reflect the levels of tetracycline resistance genes in the total microbial metagenome of the human oral and faecal microbiota.

The most common tetracycline resistance determinants in the oral microbial metagenome are *tet*(M), *tet*(Q) and *tet*(30) (cf. *tet*(M), *tet*(O) and *tet*(W) in the culture study); and are *tet*(W), *tet*(O), *tet*(Q) and *tet*(32) in the faecal microbial metagenome (cf. *tet*(M), *tet*(O) and *tet*(Q) in the culture study).

The most common erythromycin resistance genes in the oral microbial metagenome are *erm*(B), *erm*(V) and *erm*(E) (cf. *erm*(B) and *erm*(F) in the culture study); and are *erm*(B), *erm*(V) and *erm*(F) in the faecal microbial metagenome (cf. *erm*(B), *erm*(F) and *erm*(E) in the culture study).

This suggests that some determinants are more common among obligate anaerobic species and/or unculitvables.

Not all tetracycline resistance determinants express in the *E. coli* host (for example the *tet*(S) gene cloned from *E. faecium* 664:1H1), therefore construction of libraries in alternative hosts is required in order to obtain a full description of genes if a functional screen is used as the assay for their presence.

Tetracycline resistant BAC clones have been found that contain *tet*(O) from which the sequence of the genetic support is described in Chapter six; *tet*(M) supported on a Tn*916*-like element; and two rare or novel tetracycline resistance determinants, one of which appears to be supported on a chimearic element.

CHAPTER SIX

Characterisation of the *tet*(O)-Containing BAC Clone Insert

6.1 Introduction

The tetracycline resistance determinant *tet*(O) was first isolated from a *Campylobacter coli* strain in 1987 (Sougakoff *et al.*, 1987), and has since been found to have a broad host range (Table 1.4) (reviewed in Roberts, 2005).

tet(O) encodes a ribosomal protection protein of 638-640 aa (Nikolich *et al.*, 1992) which binds to the 16S ribosomal subunit at the 'A site' causing a conformational change in the 16S subunit that prevents the binding of tetracycline (Chapter 1; Section 1.1.1.2.2.2). It has varying sequence identity to other RPP genes (Table 6.1)

	% identitiy to *tet*(O)	Reference
tet(M)	76 %	Wang & Taylor, 1991
tet(W)	68 %	Barbosa *et al.*, 1999
tet(O/32/O)	76 % 100 % similarity to *tet*(O) over the first 243 nucleotides and 97.7 % identity to *tet*(O) from nucleotide 1263-1920	Melville *et al.*, 2001
tet(O/W/O) *Megasphaera elsdenii* strain 7-11	78 % 100 % similarity to the *tet*(O) gene over the first 225 nucleotides and 99.7 % similarity from nucleotide 1311-1920	Stanton & Humphrey, 2003
tet(O/W/O) *Megasphaera elsdenii* strain 14-14	72.5 % 100 % similarity to *tet*(O) over the first 243 nucleotides and 99.3 % similarity from nucleotide 1635-1920	Stanton & Humphrey, 2003

Table 6.1: Sequence similarity of *tet*(O) to other RPP and mosaic genes.

tet(O) is commonly cited as one of the most common tetracycline resistance determinants in the human microbial metagenome (Diaz-Torres *et al.*, 2006; Villedieu *et al.*, 2003). In this study it was the second most common *tet* gene in both the oral and faecal aerobic / facultative anaerobic Gram positive cultivable flora (Chapter three), and in the total faecal microbial metagenome (Chapter five). It was the third most common *tet* gene in the total oral microbial metagenome.
Its wide-spread distribution suggests it resides on one or more mobile genetic elements. The pCC31 plasmid from *Campylobacter coli* CC31 and pTet from *Campylobacter jejuni* strain 81-176 both harbour the *tet*(O) gene (Batchelor *et al.*, 2004). In addition *tet*(O) has been linked to the *tnpV* gene (Scott *et al.*, 2002), and the upstream region of the *E. coli tet*(O) has shown homology to the sequences upstream of *tet*(M) from Tn*916*, Tn*1545*, and in the recently sequenced genomes of *S. aureus*, *C. coli* and *Ureaplasma urealycum* (Wang & Taylor, 1991).

In this part of the study, NFtetC1, the tetracycline resistant BAC clone which hybridised to the *tet*(O) probe on the macroarray is sequenced in full in order to determine the genetic support of the *tet*(O) gene in this clone.

6.2 Materials and Methods

All methods used in this part of the study are described in Chapter two.

6.3 Results

6.3.1 Isolation of the *tet*(O) gene and the relatedness of the predicted Tet(O) protein to that found in *S. pneumoniae*.

A tetracycline resistant BAC clone, NFtetC1, was isolated upon functional screening of a Norwegian faecal metagenomic library, macroarray analysis showed it to harbour a *tet*(O) gene which was confirmed by PCR (Chapter five). The sequence of the isolated *tet*(O) gene was determined. The gene can encode a protein which has 99 % homology Tet(O) from *S. pneumoniae* (Table 6.2 and Figure 6.1).

Homologues of BAC*tet*(O), based on BlastX searches	Accession number	% Identity	% Similarity
tet(O) *S. pneumoniae*	CAA69103.1	98	99
tet(O) *S. Pyogenes*	YP_600745.1		98
tet(O) *E. faecalis*	AAV80411.1		
tet(O) *C. jejuni*	ZP_01072284.1		
tet(O) *Actinobacillus pleuropneumoniae*	AAY54279.1		
tet(O) *Megasphaera elsdenii*	AAR29970.1		
tet(O) *C. coli*	*YP_063396.1*	96	94

Table 6.2: Closest matches to the *tet*(O) gene from BAC clone NFtetC1. Homology is at the protein level, as determined by BlastX searches.

```
S.pneumoniae Tet(O)       MKIINLGILAHVDAGKTTLTESLLYTSGAIAEPGSVDKGTTRTDTMNLERQRGITIQTAV 60
S.pyogenes Tet(O)         MKIINLGILAHVDAGKTTLTESLLYTSGAIAEPGSVDKGTTRTDTMNLERQRGITIQTAV 45
BAC Tet(O)                MKIINLGILAHVDAGKTTLTESLLYTSGAIAEPGSVDKGTTRTDTMNLERQRGITIQTAV 60
                          ************************************************************

S.pneumoniae Tet(O)       TSFQWEDVKVNIIDTPGHMDFLAEVYRSLSVLDGAVLLVSAKDGIQAQTRILFHALQTMK 120
S.pyogenes Tet(O)         TSFQWEDVKVNIIDTPGHMDFLAEVYRSLSVLDGAVLLVSAKDGIQAQTRILFHALQTMK 105
BAC Tet(O)                TSFQWEDVKVNIIDTPGHMDFLAEVYRSLSVLDGAVLLVSAKDGIQAQTRILFHALQTMK 120
                          ************************************************************

S.pneumoniae Tet(O)       IPTIFFINKIDQEGIDLPMVYQEMKAKLSSEIIVKQKVGQHPHINVTDNDDMEQWDAVIM 180
S.pyogenes Tet(O)         IPTIFFINKIDQEGIDLPMVYQEMKAKLSSEIIVKQKVGQHPHINVTDNDDMEQWDAVIM 165
BAC Tet(O)                IPTIFFINKIDQEGIDLPMVYQEMKAKLSSEIIVKQKVGQHPHINVTDNDDMEQWDAVIM 180
                          ************************************************************

S.pneumoniae Tet(O)       GNDELLEKYMSGKPFKMSELEQEENRRFQNGTLFPVYHGSAKNNLGIRQLIEVIASKFYS 240
S.pyogenes Tet(O)         GNDELLEKYMSGKPFKMSELEQEENRKFQTGTLFPVYHGSAKNNLGIRQLKKGIASKFYS 225
BAC Tet(O)                GNDELLEKYMSGKPFKMSELEQEENRRFQNGTLFPVYHGSAKNNLGIRQLIEVIASKFYS 240
                          **************************:**.******************** : *******

S.pneumoniae Tet(O)       STPEGQSELCGQVFKIEYSEKRRRFVYVRIYSGTLHLRDVIKISEKEKIKITEMCVPTNG 300
S.pyogenes Tet(O)         STPEGQSELCGQVFKIEYSEKRRRFVYVRIYSGTLHLRDVIKISEKEKIKITEMCVPTNG 285
BAC Tet(O)                STPEGQSELCGQVFKIEYSEKRRRFVYVRIYSGTLHLRDVIKISEKEKIKITEMCVPTNG 300
                          ************************************************************

S.pneumoniae Tet(O)       ELYSSDTACSGDIVILPNDVLQLNSILGNEMLLPQRKFIENPLPMLQTTIAVKKSEQREI 360
S.pyogenes Tet(O)         ELYSSDTACSGDIVILPNDVLQLNSILGNEMLLPQRKFIENPLPMLQTTIAVKKSEQREI 345
BAC Tet(O)                ELCSSDTACSGDIVILPNDVLQLNSILGNEMLLPQRTFIENPLPMLQTTIAAKKSEQREI 360
                          ** *********************************.**************.********

S.pneumoniae Tet(O)       LLGALTEISDGDPLLKYYVDTTTHEIILSFLGNVQMEVICAILEEKYHVEAEIKEPTVIY 420
S.pyogenes Tet(O)         LLGALTEISDGDPLLKYYVDTTTHEIILSFLGNVQMEVICAILEEKYHVEAEIKEPTVIY 405
BAC Tet(O)                LLGALTEISDGDPLLKYYVDTTTHEIILSFLGKVQMEVICAILEEKYHVEAEIKEPTVIY 420
                          *********************************:**************************

S.pneumoniae Tet(O)       MERPLRKAEYTIHIEVPPNPFWASVGLSIEPLPIGSGVQYESRVSLGYLNQSFQNAVMEG 480
S.pyogenes Tet(O)         MERPLRKAEYTIHIEVPPNPFWASVGLSIEPLPIGSGVQYESRVSLGYLNQSFQNAVMEG 465
BAC Tet(O)                MERPLRKAEYTIHIEVPPNPFWASVGLSIEPLPIGSGVQYESRVSLGYLNQSFQNAVMEG 480
                          ************************************************************

S.pneumoniae Tet(O)       VLYGCEQGLYGWKVTDCKICFEYGLYYSPVSTPADFRLLSPIVLEQALKKAGTELLEPYL 540
S.pyogenes Tet(O)         VLYGCEQGLYGWKVTDCKICFEYGLYYSPVSTPADFRLLSPIVLEQALKKAGTELLEPYL 525
BAC Tet(O)                VLYGCEQGLYGWKVTDCKICFEYGLYYSPVSTPADFRLLSPIVLEQALKKAGTELLEPYL 540
                          ************************************************************

S.pneumoniae Tet(O)       HFEIYAPQEYLSRAYHDAPRYCADIVSTQVKNDEVILKGEIPARCIQEYRNDLTYFTNGQ 600
S.pyogenes Tet(O)         HFEIYAPQEYLSRAYHDAPRYCADIVSTQVKNDEVILKGEIPARCIQEYRNDLTYFTNGQ 585
BAC Tet(O)                HFEIYAPQEYLSRAYHDAPRYCADIVSTQIKNDEVILKGEIPARCIQEYRNDLTYFTNGQ 600
                          ****************************:*******************************

S.pneumoniae Tet(O)       GVCLTELKGYQPAIGKFICQPRRPNSRIDKVRHMFHKLA 639
S.pyogenes Tet(O)         GVCLTELKGYQPAIGKFICQPRRPNSRIDKVRHMFHKLA 624
BAC Tet(O)                GVCLTELKGYQPATGKLICQPRR---------------- 623
                          ************ **:******
```

Figure 6.1: Amino Acid Alignment of the NFtetC1 BAC clone *tet*(O) with closest matches in the database (*tet*(O) from *S. pneumoniae* (accession number CAA69103.1) and tet(O) from *S. pyogenes* (accession number YP_600745.1) using the ClustalW program.

6.3.2 Characterisation of the Genetic Organisation of the *tet*(O) Support

The entire insert was sequenced which produced 9.3 Kb of sequence information (1.5 Kb upstream and 5.9 Kb downstream of the *tet*(O)).

Of 11 open reading frames (ORFs) that were found (Table 6.2; Figure 6.2), six (ORFs 3, 5, 6, 7, 9 and 10) have the same direction of transcription as *tet*(O) gene.

BlastX searches were performed on these ORFs (Table 6.2).

ORF	Length of predicted protein (aa)	Closest protein match in the database	Accession Number	% identity	E value
1	51	Hypothetical protein from *C. difficile*	ZP_01229423.1	82	8e-17
2	94	Hypothetical protein from *C. difficile*	ZP_01229424.1	40	1e-06
3	56/57	Tn*916*-like (Orf6) protein from *E. faecalis* / Cpp2 from *C. jejuni*	YP_133685.1 / AAR29536.1	68 / 98	2e-17 / 2e-27
4	639	Tet(O) from *S. pneumoniae*	CAA69103.1	98	0.0
5	105	TnpV from *C. difficile*	AAF66227.1	42	9e-19
6	80	Hypothetical protein from *Rickettsia* sp.	AAL03447.1	27	1.1
7	436	Rlx-like protein from *E. faecalis*	AAF72355.1	43	4e-89
8	84	MmcQ-like protein from *Mannheimia succiniciproducens*	AAU37184.1	57	1e-24
9	197	DNA binding protein from *Listeria monocytogenes*	YP_013142.1	43	2e-09
10	231	Hypothetical transcriptional regulator from *Streptococcus thermophilus*	CAC67535.1	29	5e-18
11	414	DNA directed DNA polymerase from *C. thermocellum*	ZP_00504582	55	2e-131

Table 6.2: Homologues to the ORFs flanking *tet*(O) in the BAC clone, NFtetC1.

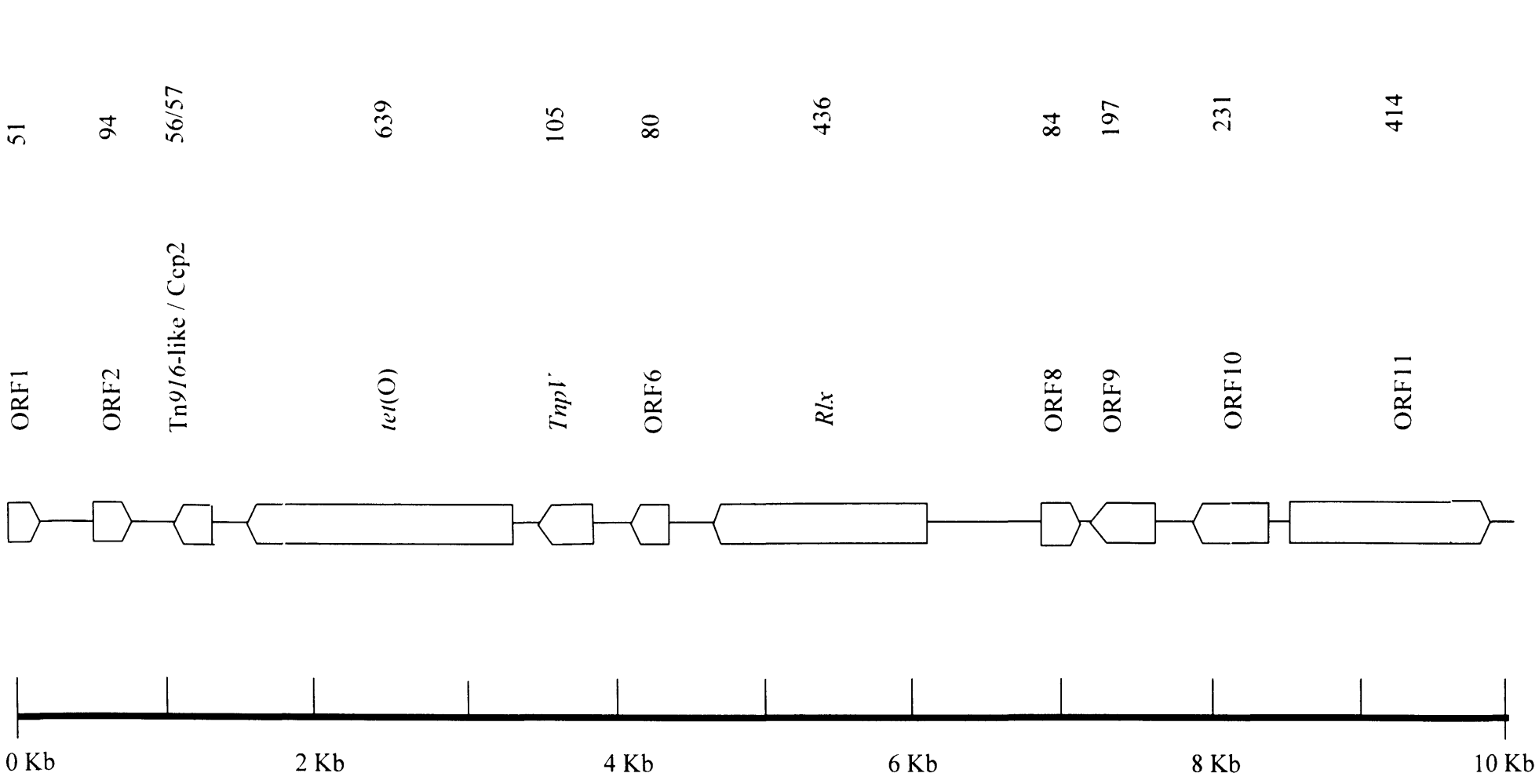


Figure 6.2: Genetic organisation of the *tet*(O) flanking DNA. Arrowed boxes represent the ORFs with proposed direction of transcription shown by the arrow. The ORFs are labelled above. The thick black line indicates scale.

6.3.3 Comparison of TnpV from NFtetC1 with those found through end-sequencing of BAC isolates

The *tet*(O) gene was immediately downstream from a gene encoding TnpV. The BAC end-sequence analysis of the English faecal library revealed six clones which exhibited amino acid sequence homology to the TnpV protein (Chapter four). The sequences were aligned (Appendix 12) and the TnpV from NFtetC1 was found to be distantly related to those from the end-sequencing approach (Figure 6.3), but closely related to the TnpV from *S. pyogenes* (Figure 6.4).

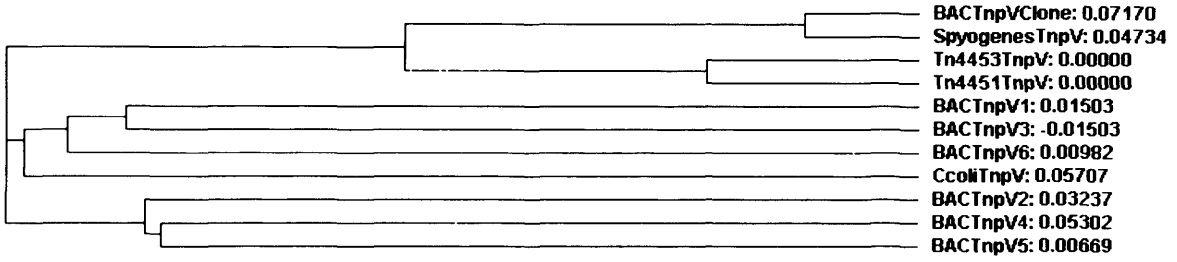


Figure 6.3 Cladogram showing the phylogenetic relationship between the translated *tnpV* genes found by end-sequencing (BACTnpV1-6), known TnpV proteins in the database and the TnpV recovered from the *tet*(O) containing BAC clone (BACTnpVclone).

```
TnpVBAC              MVQSIFEEMGGRYERQGEYILPCLTIPPEKEQSIDLFGRRHLDYLREYRKITYTNLLTSG 60
S.pyogenes TnpV      MAKSLFEELGGKYERQGDYLIPCLTVPAEEEQAIGIWGQRHLDYLKQYRKVTYTNLLTSG 60
                     *.:*:***:**:*****:*::****:*.*:**:*.::*:******::***:*********

TnpVBAC              RLNAYLADIDRQAQEHFERLIEGMKQAQGITECLKEENALEWTGRTNNIRACAREIVEKE 120
S.pyogenes TnpV      RLNAYLADIDRQAQEHFERLIEGMKQAQGITECLKEENALEWTGRTNNIRACAREIVEKE 120
                     ************************************************************

TnpVBAC              IIFA 124
S.pyogenes TnpV      IIFA 124
                     ****
```

Figure 6.4: Alignment of TnpV amino acid sequences from *S. pyogenes* and the TnpV found in the tetracycline resistant BAC clone harbouring *tet*(O) (NFtetC1). The proteins are predicted to be 88 % idendical.

6.4 Discussion

6.4.1 Sequence analysis of *tet*(O) and it's comparison to other *tet*(O) genes

The *tet*(O) gene isolated in this study shows 99% amino acid similarity to that from *S. pneumoniae*, and 98 % amino acid similarity to a number of other *tet*(O) sequences in the database (Table 6.1). The insert does not contain any genes commonly used as phylogenetic markers so it is impossible to confidently identify the original host. The sequence comparisons from one end of the insert (Figure 6.2) suggests the DNA may originate from a *Clostridium* sp., although previous studies have suggested that this end sequencing method of phylogenetic assignment is only between 57 - 96 % accurate (Nesbo *et al.*, 2005). *tet*(O) has not been previously found in *Clostridia* spp.(M Roberts, 2002). It is possible that the *tet*(O) transferred from *S. pneumoniae* to a *Clostridium* sp. on a mobile genetic element. However, it may be more likely that the gene has transferred from (or to) *S. pyogenes* (98 % homology), especially considering the adjacent *tnpV* is also closely related to the *tnpV* from *S. pyogenes* (92 % homology at the protein level).

6.4.2 Evolution of the *tet*(O) support

Mobile elements have been shown to exchange genes responsible for particular functions in modules. This can influence the host range of the element (Burrus *et al.*, 2002). The genes encoded by the clone insert that are associated with mobile elements are discussed below.

In order to obtain the complete sequence of the putative element the library will be probed with end sequences obtained from this study to obtain an ordered series of

clones covering the entire element and would enable phylogenetic markers to be discovered leading to the determination of the original host.
Furthermore, a macroarray probe, specific to this region of DNA could then be made to determine the extent to which it is spread among the faecal microbiota.

6.4.2.1 *tnpV*

The *tnpV* gene is harboured by the mobilisable transposon Tn*4451* from *C. perfringens* and Tn*4453a* and *b* from *C. difficile*. It encodes a protein of unknown function (Bannam *et al.*, 1995). In the clostridal transposons the gene overlaps by 73 bp with *tnpX*, which encodes a site-specific recombinase responsible for the precise excision of Tn*4451*-like transposons (Adams *et al.*, 2002; Lyras & Rood, 2000; Lyras *et al.*, 2004). *tnpV* has also been associated with the chloramphenicol resistance determinant *catP* in the absence of the rest of the element (Shultz *et al.*, 2003). In NFtetC1 the *tnpV* gene is immediately downstream of the *tet*(O) gene, an association previously observed by Scott (2002) in a *B. fibrisolvens* isolate. Interestingly, the *tet*(O) gene in the *B. fibrisolvens* isolate showed greatest homology to the *tet*(O) from *S. pneumoniae*, as did the NFtetC1 *tet*(O) (Table 6.1). Therefore, it is possible that the *tnpV* and *tet*(O) are transferred as one functional module, and that both elements are related to one or more as yet uncharacterised streptococcal elements.

Six translated BAC end-sequences (derived from the English faecal library) showed homology to TnpV, these sequences were not linked to *tet*(O) or other tetracycline resistance determinants, and were picked up at random. They were found to be distantly related to that from NFtetC1, suggesting the genes have diverged from the

same ancestor over time. This study illustrates that *tnpV* genes are common and can be isolated from different sources, suggesting that they are commonly located on mobile elements.

6.4.2.2 Cpp2 / Tn*916*-like Protein

Upstream from the *tet*(O) gene, *orf3* encodes a protein with 98 % homology to the Cpp2 protein from the *C. jejuni* plasmid pTet. This plasmid is closely related to the *C. jejuni* plasmid pCC31 and both harbour *tet*(O). In the plasmids the *ccp2* gene is located immediately downstream of the *tet*(O) suggesting that the *tet*(O) and *ccp2* may be transferred as part of a functional module. The *tet*(O) gene in the *C. jejuni* plasmids is identical to that from *C. jejuni* P10952 (Batchelor *et al.*, 2004). Therefore, since the BAC *tet*(O)'s closest homologue is *tet*(O) from *S. pneumoniae* (as in the *B. fibrisolvens* isolate) it is more likely the *tet*(O) is transferred with the *tnpV* rather than the *cpp2* from pTet.

Although the function of Cpp2 from pTet has yet to be determined, it shows 82 % similarity to ORF6 from Tn*916* which may be involved in mobility of the element. In this study the Cpp2-like protein harboured by the BAC clone shows 68 % similarity to theTn*916* orf6 suggesting a common ancestor for this protein and a likely function in mobility (Flannagan *et al.*, 1994).

6.4.2.3 Rlx-like Protein

Upstream from the *tnpV* gene, *orf7* encodes a protein with 43 % similarity to the Rlx-like protein from *E. faecalis*. The protein is probably required for relaxation complex

formation and plasmid mobilisation by conjugative plasmids (Garnier *et al.*, 2000). In *E. faecalis* the gene is present on the transposon Tn*1549* which harbours the *vanB2* operon for vancomycin resistance (Garnier *et al.*, 2000), therefore its presence in the BAC insert suggests it has a possible role in the mobilisation and spread of the *tet*(O) gene, and further serves to illustrate the role of the recombination between different elements in the evolution of mobile elements.

6.4.2.4 Hypothetical Transcriptional Regulator

Another putative *orf* identified in the BAC clone which has homology to genes associated with MGEs is *orf10* which has 29 % similarity to the hypothetical transcriptional regulator from *S. thermophilus*. This gene has been found on various insertion sequences including IS1191 and iso-IS981 (Guedon *et al.*, 1995), and the integrative conjugative element ICESt1 from *S. thermophilus* (Burrus *et al.*, 2000).

6.5 Conclusions

The *tet*(O) found in this study by screening metagenomic BAC libraries was found to be flanked by a number of genes associated with mobile genetic elements. This represents a new genetic support for *tet*(O).

It is likely that *tet*(O) and *tnpV* are transferred together, and that the rest of the support is a mosaic of genes from a number of different commensal and pathogenic species. Previous studies have shown the human gut to be conducive for gene transfer and the support characterised in this study may be involved in the spread of *tet*(O) throughout this environment.

CHAPTER SEVEN

Final Discussion

The threat of antibiotic resistant bacteria to public health is of great concern to the European Union and other international bodies (Degener, 1999, McCaig, 1995, Hoiby, 2000, Fish, 1995). Studies into the molecular mechanisms of resistance and their mode of spread are considered essential in order to stay one step ahead of the pathogens (Mills, 2006; Monaghan & Barrett, 2006). Such investigations, including this study, garner information on the most prevalent resistance determinants and their targets and allow the effective use of antibiotics against certain bacteria in the clinical setting (Manfedi, 2005; Sinclaire *et al.*, 2005).

The work in this study has contributed to the understanding of the prevalence of tetracycline and erythromycin resistant bacteria not only in the cultivable portion of the human oral and faecal microbiota, but also the microbial metagenome isolated from these environments.

The culture study demonstrated that tetracycline and erythromycin resistance is wide-spread among the aerobic cultivable Gram positive flora and identified the most common resistance determinants implicated. The samples were taken from healthy subjects who had not received antibiotics in the previous three months. All sample sets harboured tetracycline and erythromycin resistance determinants suggesting the genes are stable to some degree. Resistant bacteria were isolated from all countries for both samples (saliva and faecal), however, no significant differences were found in the incidence of resistance between the countries under investigation. The most likely explanation for the differences reported is sample variation.

The previously reported linkage of *tet*(M) and *int* from Tn*916* (Flanagan *et al.*, 1994) was evident from the data obtained from the array, although further analysis of individual isolates would be required to determine if these genes were harboured by the same mobile element rather than just being present in the same cell. However, the prevalence of *int* illustrates the extent to which mobile genetic elements are spread among the human microbiota. Previous studies have shown that dental plaque (Marsh, 2005; Roberts *et al.*, 2001a) and biofilms in the human intestine (Licht *et al.*, 1999) facilitate the exchange of such elements by holding bacteria in close proximity to each other.

The prevalence of different resistance determinants generally agreed with other studies of this kind (Diaz-Torres *et al.*, 2006; Lancaster *et al.*, 2003, 2005; Villedieu *et al.*, 2003; Olsvik, 1995; Walker *et al.*, 2000; Karami *et al.*, 2006). Difference could be due to the fact that this study concentrated on Gram positive organisms, and strict anaerobes were not cultured due to constraints with sample transportation and laboratory equipment. The study would be enhanced by a complete analysis of the total cultivable flora to allow a more accurate comparison between cultivable and total microbiota for each environment.

Of the tetracycline resistant Gram positive isolates, two streptococci (SStet15 and FRStet12) did not hybridise to any probes on the array. The study would benefit from an expansion of the array to include all known *tet* genes. This would eliminate the need to investigate the isolates further using PCR. This was not possible in the present study due to lack of positive controls or genes sequence in the database (*tet*(34), *tet*(35)) (preventing the design of appropriate primers). The SStet15 and

FRStet12 isolates need to be sequenced to determine the gene responsible for tetracycline resistance which could possibly be novel.

The culture study also identified a streptococcus which hybridised to the *tet*(32) probe, but not the *tet*(O) probe (isolate FStet12). All other isolates that hybridised to the *tet*(32) probe also hybridised to the *tet*(O) probe suggesting that these isolates contain mosaic genes (Stanton & Scott, 2005). The FStet12 was sub-cloned in both a Gram positive and a Gram negative host, however, no resistant colonies were found. To investigate the genetic basis of resistance in this isolate colony blots using a probe derived from a *tet*(32) PCR product should be performed. The genetic support of the gene can then also be determined. *tet*(32) was first found in a *Clostridium*-like ruminant, K10 (Melville *et al.*, 2001) and subsequent studies have found it in oral *Eubacterium* sp. and *Streptococcus* sp. isolates (Lancaster *et al.*, 2005). The genetic supports of the *tet*(32) genes were found to differ significantly and analysis of the support of the FStet12 *tet*(32) would give an insight into its origin.

The study also investigated the use of metagenomic libraries to access the uncultivable portion of the oral and faecal microbiota. It was found that ~60 % of the saliva libraries consisted of clones harbouring human DNA. No attempt was made to remove eukaryotic DNA in this study since various eukaryotic organisms have been reported in the oral microbiota. However, future studies of this kind would benefit from an attempt to remove most of the human contamination. This would cut down the amount of library required to be screened in order to gain a representative picture of the tetracycline resistance determinants present. Of the clones that did not harbour

human DNA, 74.8 % of faecal and 67.2 % of oral clones which showed homology to sequences in the database harboured DNA from anaerobic species illustrating the ability of the libraries to include sequences from organisms not found in the culture study. Furthermore, 60.7 % of faecal and 56.8 % of oral clones showed no significant similarity to any sequences in the databases, suggesting that the uncultivated portion of the microbiota is represented in each.

A study by Nesbo *et al.*, (2005) discovered that the end-sequencing and blastX homology search approach used here only gives an accurate species identification in approximately 76.5 % of clones (ranging from 57-96 % depending on species). Therefore the results are not definitive. This approach masks horizontal gene transfer since only short sequences are analysed rather than known phylogenetic markers. To improve the accuracy of clone analysis, only those clones containing phylogenetic markers such as 16S rRNA genes should be used.

The study would also benefit from similar end-sequence analysis for each library constructed. The hybridisation of total metagenomic DNA illustrated the incidence of tetracycline and erythromycin resistance determinants in the total microbiota, however, in the saliva DNA since ~60 % of DNA was human, the presence of some determinants appears to have been masked. In particular, the English samples shows a lack of *erm* genes, however, the samples from other countries exhibit higher levels of carriage. End-sequencing analysis would allow us to determine if the incidences found are truly representative or a result of the 'dilution effect' of different human DNA contents in each library. Another way to compare the incidences of resistance between countries would be to quantify the array. In this study, genes are given as a percentage hybridisation signal of the 16S rDNA control, however, different species

harbour different numbers of 16S rRNA genes (Schloss & Handelsman, 2004). In order to quantify resistance, a different control could be used such as the *sodA* gene which has been reported to occur only once in the genomes of most species (Poyart *et al.*, 1995a).

The metagenomic study highlighted the differences between the predominant *tet* and *erm* genes in the cultivable portion and total microbiota. In the oral microbial metagenome *tet*(M), *tet*(Q) and *tet*(30) are the most common (cf. *tet*(M), *tet*(O) and *tet*(W) in the culture study and differing from a similar study conducted by Diaz-Torres *et al.*, (2006) which found *tet*(M), *tet*(O) and *tet*(Q) to predominate). The most common tetracycline resistance determinants in the faecal microbial metagenome are *tet*(W), *tet*(O), *tet*(Q) and *tet*(32) (cf. *tet*(M), *tet*(O) and *tet*(Q) in the culture study). The most common erythromycin resistance genes in the oral microbial metagenome are *erm*(B), *erm*(V) and *erm*(E) (cf. *erm*(B) and *erm*(F) in the culture study); and are *erm*(B), *erm*(V) and *erm*(F) in the faecal microbial metagenome (cf. *erm*(B), *erm*(F) and *erm*(E) in the culture study). These results are important since most previous studies have only concentrated on cultivable flora. If the results from those studies are used to design clinical responses to disease, the treatment may prove ineffective if those genes that are predominant in the uncultivable portion of the microbiota are not considered.

Screening of the metagenomic libraries for tetracycline resistance resulted in 32 tetracycline resistant clones. Only 4 grew on tetracycline (10 μg/ml) after storage in glycerol stocks at −70 °C. Discussions with other groups (K. Scott personal communication 2005; A. Sundesfjord personal communication 2005) revealed

similar stability problems when using the pCC1BAC vector. However, the loss of tetracycline resistance could also be due to the carriage of the *tet* genes on highly unstable mobile elements. To establish which it is, end sequencing of the non-tetracycline resistant clones should be performed to see if any insert sequence remains. All tetracycline resistant clones should be immediately sub-cloned, and plasmids extractions performed to prevent loss of resistance determinants.

tet(S) of *E. faecium* 664:1H1 was found not to express in *E. coli*, thus not all tetracycline resistance genes can be found using this approach. Therefore, the pDL278 vector (Dunny *et al.*, 1991) was used to construct small insert metagenomic libraries in *S. mutans*. These were screened, but no tetracycline resistant clones were found, however, calculations suggest that a greater amount of library needs to be screened. This study would benefit from this since the predominant *tet* genes found in a Gram negative host could be compared to those found in a Gram positive host. It is likely that the genes found would be different demonstrating the need for different hosts when using a metagenomic approach.

Of the 4 viable tetracycline resistant BAC clones one hybridised to the *tet*(M) probe on the array, but not the *int* probe (SFtetC10). PCR analysis revealed the sequences immediately flanking the *tet*(M) gene amplified with primers specific for Tn*916*. It is possible that the element has lost the *int* gene since transferring into the original host, or that it may have acquired a different int gene. The exchange of functional modules involved in integration and conjugation can occur between different mobile elements, and this probably plays a significant part in the host specificity of the elements (Burrus *et al.*, 2002; Frost *et al.*, 2005; Osborn & Boltner, 2002). By sequencing the entire SFtetC10 insert it would be possible to characterise the mobile element and

determine if it harbours an alternative integrase gene. If so, filter mating experiments would determine its host range.

The tetracycline resistance clones IStetC1 and FRStetC11 did not hybridise to any of the probes on the array. Both were sub-cloned into pUC19, however, FRStetC11 failed to yield any tetracycline resistant colonies. End-sequence analysis of FRStetC11 revealed it to be of unknown origin. Previous studies have shown that some human DNA encoded protein conferred resistance on the host through an unknown mechanism (Diaz-Torres *et al.*, 2006), however, this can be ruled out since the entire human genome is in the database and homology searches would have revealed it to be the insert. As with the streptococcal isolates Sstet15 and FRStet12 this clone could harbour a novel resistance determinant (the *tet* genes absent from the array would need to be checked by PCR or sequencing) and would need to be fully sequenced in order to characterise the gene.

The IStetC1 BAC clone did not hybridise to any probes on the array, however, end-sequencing analysis revealed the clone to harbour what is possibly a natural chimera of two tetracycline resistance plasmids pRSB107 (accession number AP005147) and pR64 (accession number AJ851089) which both harbour *tet*(A). Sequence divergence in the probe target region could have resulted in the *tet*(A) probe not hybridising to the clone, however, the tetracycline resistance determinant may also be novel. Full sequencing of the IStetC1 insert would allow confirmation of the tetracycline resistance determinant.

The final tetracycline resistant BAC clone, NFtetC1, hybridised to the *tet*(O) probe on the array. Sequence analysis found it to be 98 % homologous to the *tet*(O) from *S.*

pneumoniae. The entire BAC insert was sequenced. Only two genetic supports of *tet*(O) have been previously characterised: pTet and pCC31 from *C. jejuni* (Batchelor *et al.*, 2004). The NFtetC1 insert was not homologous to either of these plasmids, except for the *cpp2* gene immediately upstream of the *tet*(O). *tnpV* was found downstream of *tet*(O) (this has been reported before in a *B. fibrisolvens* isolate (Scott, 2002)), and both genes are of streptococcal origin suggesting the element cloned is related to streptococcal elements.

It is unlikely that the insert contained an entire mobile element since there are no genes present that are involved in transfer, however, filter-mating studies would determine if the *tet*(O) in NFtetC1 is transferable.

Tetracycline and erythromycin resistant bacteria are present at relatively high levels in the human oral and faecal microbiota. The findings in this study further illustrate the limitations of the therapeutic use of these antibiotics. Tetracycline is widely used in the treatment of atypical pneumonia, cholera, periodontal infection, acne and many other genital, local and systemic infections (reviewed in M Roberts, 2004). Erythromycin is used against lung infections, infections of the throat and some sexually transmitted infections (reviewed in Goldman & Scaglione, 2004). Therefore the increasing incidence of resistance to these drugs over the past decades has a major effect on the treatment of the above infections.

The policies introduced to halt the spread of antibiotic resistance may not be enough to combat the problem. The carriage of resistance determinants on mobile genetic elements allows the rapid dissemination of resistance across genera barriers, and this study has proven them to be common in the absence of a selective pressure (all

samples came from individuals that have not received antibiotic therapy in the three months prior to sampling).

The study also demonstrates that most investigations up to now have not described the full picture. Investigations into resistance need to include the uncultivable portion of the microbiota. Studies such as this are essential in order to identify the molecular basis of resistance, and the mechanisms of transfer of resistance genes. This information can then be used to investigate potential novel drug targets, and to groom the clinical response appropriately.

References

1. **Aarestrup FM, Seyfarth AM, Emborg HD, Pedersen K, Hendriksen RS, Bager F.** 2001. Association between the use of avilamycin for growth promotion and the occurrence of resistance among *Enterococcus faecium* from broilers: epidemiological study and changes over time. Microb. Drug. Resist. 6(1): 71-75.

2. **Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE.** 2005. Defining the normal bacterial flora of the oral cavity. J. Clin. Microbiol. 43(11) : 5721-5732.

3. **Abascal F, Valencia A.** 2002. Clustering of proximal sequence space for the identification of protein families. Bioinformatics. 18(7) : 908-921.

4. **Abbani M, Iwahara M, Clubb RT.** 2005. The structure of the excisionase (Xis) protein from conjugative transposon Tn*916* provides insights into the regulation of heterobivalent tyrosine recombinases. J. Mol. Biol. 347(1):11-25.

5. **Abraham LJ, JI Rood.** 1987. Identification of Tn*4451* and Tn*4452*, chloramphenicol resistance transposons from *Clostridium perfringens*. J. Bacteriol. 169(4):1597-1584

6. **Adams V, Lyras D, Farrow KA, and Rood JI.** 2002. The clostridial mobilisable transposons. Cell Mol. Life Sci. 59(12):2033-43.

7. **Addy M, Slayne MA, and Wade WG.** 1992. The formation and control of dental plaque - an overview. J. Appl. Bacteriol. 73(4):269-78.

8. **Agerso Y, Jensen LB, Givskov M, Roberts MC.** 2002. The identification of a tetracycline resistance gene *tet*(M) on a Tn*916*-like transposon, in the *Bacillus cereus* group. FEMS Microbiol. Lett. 214: 251-256.

9. **Agerso Y, D Sanvang.** 2005. Class 1 integrons and tetracycline resistance genes in *Alcaligenes*, *Arthrobacter*, and *Pseudomonas* spp. Isolated from pigsties and manured soil. Appl. Environ. Microbiol. 71(12):7941-7947.

10. **Agerso Y, Pedersen AG, Aarstrup FM.** 2006. Identification of Tn*5397*-like and Tn*916*-like transposons and diversity of the tetracycline resistance gene *tet*(M) in enterococci from humans, pigs and poultry. J. Antimicrob. Chemother. 57(5) ; 832-839.

11. **Alduina R, De Grazia S, Dolce L, Salerno P, Sosio M, Donadio S, Puglia AM.** 2003. Artificial chromosome libraries of *Streptomyces coelicolor* A3(2) and *Planobispora rosea*. FEMS Microbiol. Lett. 218: 181-186.

12. **Alexander HE, Damrosch DS, Ellis.** 1956. Correlation of in vitro susceptibility of tubercle bacilli in patients with miliary and meningeal tuberculosis with their response to therapy. Ann. Rev. Tuberc. 74(2 part 2): 232-240.

13. **Allard JD, KP Bertrand.** 1993. Sequence of a class E tetracycline resistance gene from *Escherichia coli* and comparison of related tetracycline efflux pumps. J. Bacteriol. 175(14): 4554-4560.

14. **Allignet J, N El Solh.** 1997. Characterisation of a new staphylococcal gene *vgaB* encoding a putative ABC transporter conferring resistance to streptogramin A and related compounds. Gene. 202(1-2): 133-138.

15. **Alpert CA, Mater DD, Muller MC, Ouriet MF, Duval-Iflah Y, Corthier G.** 2003. Worst case scenarios for horizontal gene transfer from *Lactococcus lactis* carrying heterologous genes to *Enterococcus faecalis* in the digestive tract of gnotobiotic mice. Environ. Biosafety. Res. 2(3): 173-180.

16. **Altschul SF, Gish W, Miller W, Myers EW, and Lipman DJ.** 1990. Basic local alignment search tool. J. Mol. Biol. 215:403-410.

17. **Aminov RI, Garrigues-Jeanjean N, Mackie RI.** 2001. Molecular ecology of tetracycline resistance: development and validation of primers for detection of tetracycline resistance genes encoding ribosomal protection protein. Appl. Environ. Microbiol. 67(1): 22-32.

18. **Andrews JM,** 2001. BSAC Standardized Disc Susceptibility Testing Method. J. Antimicrob. Chemother. 48:S43-57.

19. **Ankenbauer RG.** 1997. Reassessing forty years of genetic doctrine: retrotransfer and conjugation. Genetics. 145:543-549.

20. **Arpin C, Canron MH, Noury P, Quentin C.** 1999. Emergence of *mef*A and *mef*E genes in beta-haemolytic streptococci and pneumococci in France. J. Antimicrob. Chemother. 44(1): 133-124.

21. **Arthur M, Autissier D, Courvalin P.** 1986. Analysis of the nucleotide sequence of the *ereB* gene encoding the erythromycin esterase type II. Nucleic Acids Res. 14(12): 4987-4999.

22. **Arthur M, Andremont A, Courvalin P.** 1987. Distribution of erythromycin esterase and rRNA methylase genes in members of the family Enterobacteriaceae highly resistant to erythromycin. Antimicrob. Agents Chemother. 31(3): 404-409.

23. **Arthur M, Molinas C, Depardieu F, Courvalin P.** 1993. Characterisation of Tn*1546*, a Tn3-related transposon conferring glycopeptide resistance by synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. J. Bacteriol. 175(1): 177-127.

24. **August PR, Grossman TH, Minor C, Draper MP, MacNeil IA, Pemberton JM, Call KM, Holt D, Osburne MS.** 2000. Sequence analysis and functional characterisation of the violacein biosynthetic pathway from *Chromobacterium violaceum*. J. Mol. Microbiol. Biotechnol. 2(4): 513-519.

25. **Austin DJ, Kristinsson KG, Anderson RM.** 1999. The relationship between the volume of antimicrobial consumption in human communities and the frequency of resistance. Proc. Natl. Acad. Sci. U S A. 296(3):1152-6.

26. **Avrain L, Vernozy-Rozand C, Kempf I.** 2004. Evidence for natural horizontal transfer of *tet*(O) gene between *Campylobacter jejuni* strains in chickens. J. Appl. Microbiol. 97(1): 134-140.

27. **Backhed F, Ding H, Wang T, Hooper LV, Koh GY, Nagy A, Semenkovich CF, Gordon JI.** 2004. The gut microbiota as an environmental factor that regulates fat storage. PNAS. 101(44):15718-15723.

28. **Bahl MI, Sorensen SJ, Hansen LH, Licht TR.** 2004. Effect of tetracycline on transfer and establishment of the tetracycline inducible conjugative transposon Tn*916* in the guts of gnotobiotic rats. Appl. Environ. Microbiol. 70(2):758-764.

29. **Baker-Austin C, Wright MS, Stepanauskas R Mc Arthur JV.** 2006. Co-selection of antibiotic and metal resistance. Trends Microbiol. 2006: marII.

30. **Balbu HJ.** 2004. Chloramphenicol. A review. Pediatr. Rev. 25(8):284-288.

31. **Ball KD, JT Trevors.** 2002. Bacterial genomics: the use of DNA microarrays and bacterial artificial chromosomes. J. Microbiol. Methods. 49: 275-284.

32. **Banerjee A, Yaseri M, Munson M.** 2002. A Method for the detection and quantification of bacteria in human carious dentine using fluorescent in situ hybridisation. J. Dent. 30(7-8): 359-363.

33. **Bannam TL, Crellin PK, Rood JI.** 1995. Molecular genetics of the chloramphenicol resistance transposon Tn*4451* from *Clostridium perfingens* ; the TnpX site-specific recombinase excises a circular transposon molecule. Mol. Microbiol. 16(3) : 535-551.

34. **Baquero F, Baquero-Artigao G, Canton R, Garcia-Rey C.** 2002. Antibiotic consumption and resistance selection in *Streptococcus pneumoniae*. J. Antimicrob. Chemother. 50 (suppl S2): 27-37.

35. **Barbosa TM, Scott KP, and Flint FJ.** 1999. Evidence for recent intergenic transfer of a new tetracycline resistance gene, *tet*(W), isolated from *Butytivibrio fibrisolvens*, and the occurrence of *tet*(O) in ruminal bacteria. Environ. Microbiol. 1:53-64.

36. **Barbosa TM, SB Levy.** 2000. The impact of antibiotic use on resistance development and persistence. Drug Res. Updates. 3(5): 303-311.

37. **Bartosch S, Fite A, Macfarlane GT, McMurdo MET.** 2004. Characterization of bacterial communities in feces from healty elderly volunteers and hositalized elderly patients by using real-time PCR and effects of antibiotic treatment on the fecal microflora. Appl. Env. Microbiol. 70(6):3575-3581.

38. **Batchelor RA, Pearson BM, Friis LM, Guerry P, Wells JM.** 2004 Nucleotide sequences and comparison of two large conjugative plasmids from different *Campylobacter* species. Microbiology. 150(pt 10):3507-3517.

39. **Beighton D.** 2005 The complex oral microflora of high-risk individuals and groups and its role in the caries process. Community Dent. Oral Epidemiol. 33(4):248-255.

40. **Beja O, Suzuki MT, Koonin EV, Aravind l, Hadd A, Nguyen LP, Villacorta R, Amjadi M, Garrigues C, Jovanovich SB, Feldman RA, DeLong EF.** 2000. Construction and analysis of bacterial artificial chromosome libraries from a marine microbial assemblage. Env. Microbiol. 2(5):516-529.

41. **Beja O, Aravind L, Koonin EV, Suzuki MT, Hadd A, Nguyen LP, Jovanovich SB, Gates CM, Feldman RA, Spudich JL, Spudich EN, DeLong EF.** 2000. Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. Science. 289(5486): 1902-1906.

42. **Bennett PM.** 1999. Integrons and gene cassettes: a genetic construction kit for bacteria. J. Antimicrob. Chemother. 43:1-4.

43. **Bennett PM.** 2004. Genome plasticity: insertion sequence elements, transposons and integrons and DNA rearrangements. Methods Mol. Biol. 266:71-113.

44. **Bentorcha F, Clermont D, de Cespedes G, Horaud T.** 1992. Natural occurrence of structures in oral streptococci and enterococci with DNA homology to Tn*916*. Antimicrob. Agents Chemother. 36(1):59-63.

45. **Berry AE, Chiocchini C, Selby T, Sosio M, Wellington EMH.** 2003. Isolation of high molecular weight DNA from soil for cloning into BAC vectors. FEMS Microbiol. Lett. 223(1): 15-20.

46. **Bertram J, P Durre.** 1989. Conjugal transfer and expression of streptococcal transposons in *Clostridium acetobutylicum*. Archives Microbiol. 151(6) : 551-557.

47. **Bertram J, Stratz M, Durre P.** 1991. Natural transfer of conjugative transposon Tn*916* between Gram positive and Gram negative bacteria. J. Bact. 173(2):443-448.

48. **Betriu C, Culebras E, Redondo M, Rodriguez-Avail I, Gomez M, Boloix A, Picazo JJ.** 2002. Prevalence of macrolide and tetracycline resistance mechanisms in *streptococcus pyogenes* isolates and in vitro susceptibility to telithromycin. J. Antimicrob. Chemother. 50:433-442.

49. **Bevan MW, MD Chilton.** 1982. T-DNA of the Agrobacterium Ti and Ri plasmids. Ann. Rev. Genetics. 16 : 357-384.

50. **Biavasco F, Miele A, Vignaroli C, Manso E, Lupidi R, Varaldo PE.** 1996. Genotypic characterisation of a nosocomial outbreak of VanA *Enterococcus faecalis*. Microb. Drug Resist. 2(2) : 231-237.

51. **Bik EM, Eckberg PB, Gill SR, Nelson KE, Purdom EA, Francois F, Perez-Perez G, Blaser MJ, Relman DA.** 2006. Molecular analysis of the bacterial microbiota in the human stomach. PNAS. 103(3): 732-737.

52. **Blackwood RK.** 1985. Structure determination and total synthesis of the tetracyclines. P59-136 in JJ Hlavka and JH Boothe (ed.) Handbook of experimental pharmacology vol 78. Springer-Verlag KG, Berlin.

53. **Blake DP, Humphry RW, Scott KP, Hillman K, Fenion DR, Low JC.** 2003. Influence of tetracycline exposure on tetracycline resistance and the carriage of tetracycline resistance genes within commensal *Escherichia coli* populations. J. Appl. Microbiol. 94: 1087-1097.

54. **Blaut M, Collins MD, Welling GW, Dore J, van Loo J, de Vos W.** 2002. Molecular biological methods for studying the gut microbiota: the EU human gut flora project. Brit. J. of Nutrition. 87, suppl. 2:S203-S211.

55. **Boneca IG, G Chiosis.** 2003. Vancomycin resistance; occurrence, mechanisms and strtergies to combat it. Expert. Op. Therapeut. Targets. 7(3):311-328.

56. **Bonten MJ, Willems R, Weinstein RA.** 2001. Vancomycin-resistant enterococci: why are they here, and where do they come from? Lancet Infect. Dis.1(5):314-25.

57. **Bosnar M, Kelnerie Z, Munic V, Erakovic V, Parnham MJ.** 2005. Cellular uptake and efflux of azithromycin, erythromycin, clarithromycin, telithromycin and cethromycin. Antimicrob. Agents Chemother. 49(6) : 2372-2377.

58. **Boudewijns M, Magerman K, Verhaegen J, Debrock G, Peetermans WE, Donkersloot P, Mewis A, Peeters V, Rummens JL, Cartuyvels R.** 2003. Rothia dentocariosa, endocarditis and mycotic aneurysms: case report and review of the literature. Clin. Microbiol. Infect. 9:222-229.

59. **Bouma JE, RE Lenski.** 1988. Evolution of a bacteria-plasmid association. Nature. 335 : 351-352.

60. Bourgeouis-Nicolas N *et al.* 2006. Comparitive study of *vanA* gene transfer from *Enterococcus faecium* to *Enterococcus faecalis* and to *E. facium* in the intestine of mice. FEMS Microbiol. Lett. 254(1):27-33.

61. **Bourlioux P, Koletzko B, Guarner F, Braesco V.** 2003. The intestine and its microflora are partners for the protection of the host. Am. Soc. Clin. Nutr. 78:675-683.

62. **Bowden GH.** 1997. Does assessment of microbial composition of plaque/saliva allow for diagnosis of disease activity of individuals? Community Dent. Oral Epidemiol. 25(1):76-81.

63. **Brady SF, Chao CJ, Clardy J.** 2004 Long chain N-Acetyrosine synthases from environmental DNA. Appl. Environ. Microbiol. 70(11): 6865-6870.

64. **Breitbart M, Hewson I, Felts B, Mahaffy JM, Nulton J, Salamon P, Rohwer F.** 2003. Metagenomic analysis of an uncultured viral community from human feces. J. Bacteriol. 185(20):6220-6223.

65. **Brodersen DE, Clemons WM, Carter AP, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V.** 2000. The structural basis for the action of the antibiotics

tetracycline, pactamycin and the hygromycin B on the 30S ribosomal subunit. Cell. 103:1143-154.

66. **Bruno WJ, Knill E, Balding DJ, Bruce DC, Doggett NA, Sawhill WW, Stallings RL, Whittaker CC, Torney DC.** 1995. Efficient pooling designs for library screening. Genomics. 26(1):21-30.

67. **Broson JJ, JF Barrett.** 2001. Quinolone, everninomycin, glycylcyclines, carbaenem, lipopeptide and cephem antibacterials in clinical development. Curr. Med. Chem. 8(14): 1775-1793.

68. **Bronzwaer S Cars O, Buchholz U, Molstad S, Goettsch W, Veldhuijzen IK, Kool JL, Sprenger MJW, Degener JE.** 2002. A European study on the relationship between antimicrobial use and antimicrobial resistance. Emerg. Infect. Dis. 8(3): 278-282

69. **Bronzwaer S, Buchholz U, Courvalin P, Snall J, Conaglia G, de Neeling A, Aubry-Damon H, Degener J, EARSS participants.** 2002 Comparability of antimicrobial susceptibility test results from 22 European countries and Israel. J. Antimicrob. Chemother. 50(6): 953-964

70. **Bronzwaer S, Lonnroth A, Haigh R.** 2004. The European Community stratergy against antimicrobial resistance. Euro. Surveillance. 9(1):30-34

71. **Broskey J, Coleman K, Gwynn MN, McCloskey L, Traini C, Voelker L, Warren R.** 2000. Efflux and target mutations as quinolone resistance mechanisms in clinical isolates of *Steptococcus pneumoniae*. J. Antimicrob. Chenother. 45 (suppl S1): 95-99.

72. **Bryskier A, MT Labro.** 1994. Macrolides: new therapeutic prospects. Presse Med. 23(38): 1762-1766.

73. **Bryson V, Demerec M.** 1950. Patterns of resistance to antimicrobial agents. Ann. NY Acad. Sci. 53(2): 283-289.

74. **Bryskier A.** 2002. Viridans group streptococci: a reservoir of resistant bacteria in oral cavities. Clin. Microbiol. Infect. 8(2):65-9.

75. **Buck MA, BS Cooperman.** 1990. Single protein omission reconstitution studies of tetracycline binding to the 30S subunit of *Escherichia coli* ribosomes. Biochem. 29(22) : 5374-5379.

76. **Burdet V.** 1991. Purification and characterisation of Tet(M), a protein that renders ribosomes resistant to tetracycline. J. Biol. Chem. 266(5):2872-2877.

77. **Burrus V, Roussel Y, Decaris B, Guedon G.** 2000. Characterisation of a novel integrative element. ICESt1 in the lactic acid bacterium *Streptococcus thermophilus*. Appl. Environ. Microbiol. 66(4): 1749-1753.

78. **Burrus V, Bontemps C, Decaris B, Guedon G.** 2001. Characterisation of a novel type II restriction modification system Sth3681, encoded by the integrative element ICESt1 of *Streptococcus thermophilus* CNRZ368. Appl. Environ. Microbiol. 67(4) :1522-1528.

79. **Burrus V, Pavlovic G, Decaris B, Guedon G.** 2002. Conjugative transposons: the tip of the iceberg. Mol. Microbiol. 46(3):601-10.

80. **Butaye P, Devriese LA, Haesebrouck F.** 2001. Differences in antibiotic resistance patterns of *Enterococcus faecalis* and *Enterococcus faecium* strains isolated from farm and pet animals. Antimicrob. Agents Chemother. 45(5):1374-1378.

81. **Bywater R, McConville M, Phillips I, Shryock T.** 2005. The susceptibility to growth promoting antibiotics of *Enterococcus faecium* isolates from pigs and chickens in Europe. J. Antimicrob. Chemother. 56(3): 538-543.

82. **Calva JJ, Sifuentes-Osornio J, Ceron C.** 1996. Antimicrobial resistance in fecal flora: longitudinal community based surveillence of children from urban Mexico. Antimicrob. Agents Chemother. 40(7): 1699-1702.

83. **Call DR, Bakko MK, Krug MJ, Roberts MC.** 2003. Identifying antimicrobial resistance genes with DNA microarrays. Antimicrob. Agents Chemother. 47(10): 3290-3295.

84. **Campbell A, Starlinger P, Berg DE, Botstein D, Lederberg EM, Novick RP, Szybalski W.** 1979. Nomenclature of transposable elements in Prokaryotes. Plasmid. 2 : 466-473.

85. **Canchaya C, Fournous G, Chibani-Chennoufi S, Dillmann ML, and Brüssow H.** 2003. Phage as agents of lateral gene transfer. Curr. Opin. Microbiol. 6:417-424.

86. **Canchaya C, Fournous G, Brussow H.** 2004. The impact of prophages on bacterial chromosomes. Mol. Micro. 53(1):9-18.

87. **Carias LL, Rudin SD, Donskey CJ, Rice LB.** 1998. Genetic linkage and cotransfer of a novel *vanB*-containing transposon (Tn5382) and a low-affinity penicillin-binding protein 5 gene in a clinical vancomycin-resistant *Enterococcus faecium* isolate. J. Bacteriol. 180(17): 4426-4434

88. **Castagliuolo I, Riegler MF, Valenick L, LaMont T, Pothoulakis C.** 1999. *Saccharomyces boulardii* protease inhibits the effects of *Clostridium diffficile* toxins A and B in human colonic mucosa. Infect. Immun. 67(1):302-307.

89. **Casewell M, Friis C, Marco E, McMullin P, Phillips I.** 2003. The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. J. Antimicrob. Chemother. 52: 159-161.

90. **Celli J, Trieu-Cuot P.** 1998. Circularization of Tn*916* is required for expression of the transposon-encoded transfer functions: characterization of long tetracycline-inducible transcripts reading through the attachment site. Mol. Microbiol. 28(1):103-17.

91. **Chain E, Florey HW, Gardner AD, Heatley NG, Jennings MA, Orr-Ewing J, Sanders AG.** 2005. The classics: penicillin as a chemotherapeutic agent. Clin. Orthopaedics Res. 439: 23-26.

92. **Chalmers R, Sewitz S, Lipkow K Crellin P.** 2000. Complete nucleotide sequence of Tn*10*. J. Bacteriol. 182(10):2970-2.

93. **Chen I, D Dubnau.** 2004. DNA uptake during bacterial transformation. Nat. Revs. Microbiol. 2 : 241-245.

94. **Chen I, Christie PJ, Dubnau D**. 2005. The ins and outs of DNA transfer in bactetria. Science. 310(5753): 1456-1460.

95. **Chen K, L Pachter.** 2005. Bioinformatics for whole-genome shotgun sequencing of microbial communities. PloS Comp. Biol. 1(2): 106-112.

96. **Chopra I, Roberts M.** 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiol. Mol. Biol. Rev. 65(2):232-60

97. **Chopra I.** 2002. New developments in tetracycline antibiotics: glycylcyclines and tetracycline efflux pump inhibitors. Drug Resist. Update. 5(3-4):119-125.

98. **Christensen NM, Axelsen KB, Nicolaisen M, Schulz A**. 2005. Phytoplasmas and their interactions with hosts. Trends Plant Sci. 10(11): 1360-1385.

99. **Christie PJ, JP Vogel.** 2000. Bacterial type IV secretion: conjugal systems adapted to deliver effector molecules to host cells. Trends Microbiol. 8(8): 354-360.

100. **Christie PJ.** 2001. Type IV secretion: intercellular transfer of macromolecules by systems ancestrally related to conjugation machines. Mol. Microbiol. 40(2):294-305.

101. **Chung WO, Persson GJ, Roberts MC**. 2002. Distribution of *erm*(F) and *tet*(Q) genes in 4 oral bacterial species and genotypic variation between resistant and susceptible isolates. J. Clin. Periodontol. 29:152-158.

102. **Cizman M, Beovic B, Krcmery V, Barsic B, Tamm E, Ludwig E, Pelemis M, Karovski K, Grzesiowski P, Gardovska D, Volokha A, Keuleyan E, Stratchounski L, Dumitru C, Titov LP, Usonis V, Dvorak P.** 2004. Antibiotic policies in central eastern Europe. Int. J. Antimicrob. Agents. 24(3): 199-204.

103. **Clancy J, Petitpas J, Dib-Hajj F, Yuan W, Cronan M, Kamath AV, Bergeron J, Retsema JA.** 1996. Molecular cloning and functional analysis of a

novel macrolide-resistance determinant, *mef*A, from *Streptococcus pyogenes*. Mol. Microbiol. 22(5) : 867-879.

104. **Clarridge JE.** 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin. Microbiol. Rev. 17(4):840-862.

105. **Clewell DB, Flannagan SE, Jaworski DD.** 1995. Unconstrained bacterial promiscuity: the Tn*916*-Tn*1545* family of conjugative transposons. Trends Microbiol. 3(6):229-36.

106. **Climo MW, Sharma VK, Archer GL.** 1996. Identification and characterisation of the origin of conjugative transfer (oriT) and a gene (nes) encoding a single stranded endonuclease on the staphylococcal plasmid pGO1. J. Bacteriol. 178(16): 4975-4983.

107. **Collis CM, RM. Hall.** 1992. Site-specific deletion and rearrangement of integron insert genes catalyzed by the integron DNA integrase. J. Bacteriol. 174(5):1574-85.

108. **Collis CM, RM Hall.** 1995. Expression of antibiotic resistance genes in the integrated cassettes of integrons. Antimicrob. Agents Chemother. 39(1):155-162.

109. **Connell SR, Trieber CA, Stetzi U, Einfeldt E, Taylor DE, Nierhaus KH.** 2002. The tetracycline resistance protein Tet(O) perturbs the conformation of the ribosome decoding centre. Mol. Micro. 45(6):1463-1472.

110. **Connell SR, Trieber CA, Dinos GP, Einfeldt E, Taylor DE, Nierhaus KH.** 2003. Mechaism of Tet(O)-mediated tetracycline resistance. EMBO. 22(4):945-953.

111. **Connell SR, Tracz DM, Nierhaus KH, Taylor DE**. 2003. Ribosomal protection proteins and their mechanism of tetracycline resistance. Antimicrob. Agents. Chemother. 47(12):3675-3681.

112. **Cooper AJ, Shoemaker NB, Salyers AA.** 1996. The erythromycin resistance gene from the Bacteroides conjugal transposon TcR EmR 7853 is nearly identical

to *ermG* from *Bacillus sphaericus*. Antimicrob. Agents. Chemother. 40(2):506-508.

113. **Corda M.** 1950. Observations on chloramphenicol resistance induced in vitro in a typhoid strain. Soc. Ital. Biol. Sper. 26(5): 798-800.

114. **Corpet DE.** 1992. Antibiotic residues and digestive microflora. Ann. Gastroenterol. Hepatol. 28(5):235-238.

115. **Costerton JW, Ellis B, Lam K, Johnson F, Khoury AE.** 1994. Mechanism of electrical enhancement of efficacy of antibiotics in killing biofilm bacteria. Antimicrob. Agents Chemother. 38(12): 2803-2809.

116. **Costerton JW, Stewart PS, Greenberg EP.** 1999. Bacterial biofilms: a common cause of persistant infections. Science 284(5418):1318-1322

117. **Courtois S, Cappellano CM, Ball M, Francou FX, Normand P, Helynck G, Martinez A, Kolvek SJ, Hopke J, Osburne MS, August PR, Nalin R, Guerineau M, Jeannin P, Simonet P, Pernodet JL.** 2003. Recombinant environmental libraries provide access to microbial diversity for drug discovery from natural products. Appl. Environ. Microbiol. 69(1): 49-55.

118. **Cousin S, Whittington WLH, Roberts MC.** 2003. Acquired macrolide resistance genes in pathogenic *Neisseria* Spp. Isolated between 1940 and 1987. Antimicrob. Agents Chemother. 47(12):3877-3880.

119. **Crellin PK. and JI. Rood.** 1998. Tn*4451* from *Clostridium perfringens* is a mobilizable transposon that encodes the functional Mob protein, TnpZ. Molecular Microb. 27(3):631.

120. **Dahlberg C, L Chao.** 2003. Amelioration of the cost of conjugative plasmid carriage in Escherchia coli K12. Genetics. 165(4): 1641-1649.

121. **Dailidiene D, Bertoli MT, Miciuleviciene J, Mukhopadhyay AK, Dailide G, Pascasio MA, Kupcinskas L, Berg DE.** 2002. Emergence of tetracycline resistance in *Helicobacter pylori*: multiple mutational changes in 16S ribosomal DNA and other genetic loci. Antimicrob. Agents Chemother. 46(12):3940-6.

122. **Daley M, Knauf KR, Summerfelt KR, Turner JC.** 1998. Co-transformation with one *Agrobacterium tumefaciens* strain containing two binary plasmids as a method for producing marker-free transgenic plants. Plant Cell Reports. 17(6-7): 489-496.

123. **Dantley KA, Dannelly HK, Burdett V.** 1998. Binding interaction between Tet(M) and the ribosome: Requirements for binding. J. Bacteriol. 180(16):4089-4092.

124. **Davey ME, GA O'Toole.** 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. 64(4):847-867.

125. **Davies J.** 1994. Inactivation of antibiotics and dissemination of resistance genes. Science. 264(5157):375-382

126. **Davies J.** 1994. New pathogens and old resistance genes. Microbiologia. 10(1-2):9-12

127. **Davis IJ, Roberts AP, Ready D, Richards H, Wilson M, Mullany P.** 2005. Linkage of a novel mercury resistance operon with streptomycin resistance on a conjugative plasmid in *Enterococcus faecium*. Plasmid. 54(1): 26-38.

128. **De La Cochetiere M, Durand T, Lepage P, Bourreille A, Galmiche JP, Dore J.** 2005. Resilience of the dominant human faecal microbiota upon short-course antibiotic challenge. J. Clin. Microbiol. 43(11) 5588-5592.

129. **De Leener E, Martel A, Decostere A, Haesebrouck F.** 2004. Distribution of the *erm*(B) gene, tetracycline resistance genes and Tn*1545*-like transposons in macrolide and licosamide resistant enterococci from pigs and humans. Microb. Drug Resist. 10(4):341-345.

130. **Delgado S, Florez AB, Mayo B.** 2005. Antibiotic susceptibility of Lactobacilus and Bifidobacterium species from the human gastrointestinal tract. Curr. Microbiol. 50(4):202-207.

131. **Derbyshire KM, Hwang L, Grindley ND.** 1987. Genetic analysis of the interaction of insertion sequence IS903 transposase with its terminal inverted repeats. Proc. Natl. Acad. Sci. USA. 84: 8049-8053.

132. **De Stasio EA, Moazed D, Noller HF, Dahlberg AE.** 1989. Mutations in 16S ribosomal RNA disrupt antibiotic-RNA interactions. EMBO. 8(4):1213-1216.

133. **Diaz-Torres ML, McNab R, Spratt DA, Villedieu A, Hunt N, Wilson M, and Mullany P.** 2003. Novel tetracycline resistance determinant from the oral metagenome. Antimicrob. Agents Chemother. 47:1430-1432.

134. **Diaz-Torres ML, Villedieu A, Hunt N, McNab R, Spratt DA, Allan E, Mullany P, Wilson M.** 2006. Determining the antibiotic resistance potential of the indigenous oral microbiota of humans using a metagenomic approach. FEMS Microbiol. Lett. 258 : 257-262.

135. **Dick LK, Bernhard AE, Brodeur TJ, Santo Domingo JW, Simpson JM, Walters SP, Field KG.** 2005. Host distributions of uncultivated fecal bacteroidales bacteria reveal genetic markers for fecal source identification. Appl. Env. Microbial. 71(6):3184-3191.

136. **Di Conza J, Ayala JA, Porto A, Mollerach M, Gutkind G.** 2005. Molecular characterisation of InJR06, a class I integron located in a conjugative plasmid of Salmonella enetrica serovar *Thyphimurium*. Int. Microbiol. 8(4) : 287-290.

137. **Domingo MC, Huletsky A, Giroux R, Boissinot K, Picard FJ, Lebel P, Ferraro MJ, Bergeron MG.** 2005. High prevalence of glycopeptide resistance genes *van*B, *van* D and *van*G not associated with enterococci in human fecal flora. Antimicrob. Agents Chemother. 49(11): 4784-4786.

138. **Doolittle WF.** 1999. Phylogenetic classification and the universal tree. Science. 284(5423):2124-2129.

139. **Dreiseikelmann B.** 1994. Translocation of DNA across bacterial membranes. Microbiol. Rev. 58(3):293-316.

140. **Drews TD, Temte JL, Fox BC.** 2006. Community associated methicillin resistant *Staphylococcus aureus*: review of an emerging public health concern. WMJ. 105(1):52-57.

141. **Drouault S, Corthier G, Ehrlich SD, Renault P.** 1999. Survival, physiology and lysis of *Lactococcus lactis* in the digestive tract. Appl. Environ. Microbiol. 65(11) : 4881-4886.

142. **Dubnau D.** 1991. Genetic competence in *Bacillus subtilis*. Microbiol. Rev. 55(3):395-424.

143. **Dubnau D.** 1999. DNA uptake in bacteria. Annu. Rev. Microbiol. 53:217–44.

144. **Duncan SH, Scott KP, Ramsay AG, Harmsen HJM, Welling GW, Stewart CS, Flint HJ.** 2003. Effects of alternative dietary substances on competition between human colonic bacteria in an anaerobic fermentor system. Appl. Env. Microbiol. 69(2):1136-1142.

145. **Dunny GM, Lee LN, LeBlanc DJ.** 1991. Improved electroporation and cloning vector system for Gram positive bacteria. Appl. Environ. Microbiol. 57(4): 1194-1201.

146. **Dutta C, A Pan.** 2002. Horizontal gene transfer and bactetial diversity. J. Biosci. 27(1) :27-33.

147. **Easter CL, Schwab H, Helinski DR.** 1998. Role of the *parCBA* operon pf the broad-host-range plasmid RK2 in stable plasmid maintenance. J. Bacteriol. 180(22): 6023-6030.

148. **Eaton TJ, MJ Gasson.** 2001. Molecular screening of Enterococcus virulence determinants and potential for genetic exchange between food and medical isolates. Appl. Environ. Microbiol. 67(4) :1628-1635.

149. **Eckburg PB, Bik EM, Bernstein CN, Purdom E, Dethlefsen L, Sargent M, Gill SR, Nelson KE, Relman DA.** 2005. Diversity of the human intestinal microbial flora. Science. 308(5728) :1635-1638.

150. **Edlund C and CE Nord.** 2000. Effects on the human normal microflora of oral antibiotics for treatment of urinary tract infections. J. Antimicrob. Chemother. 46 suppl S1: 41-48.

151. **Edwards CA, AM Parrett.** 2002. Intestinal flora during the first months of life; new perspectives. Br. J. Nutr. 88 suppl1: S11-18.

152. **Entcheva P, Liebl W, Johann A, Hartsch T, Streit WR.** 2001. Direct cloning from enrichment cultures, a reliable stratergy for isolation of complete operons and genes from microbial consortia. Appl. Env. Microbiol. 67(1):89-99.

153. **Fanaro S, Chierici R, Guerrini P, Vigi V.** 2003. Intestinal microflora in early infancy; composition and development. Acta. Paediatr. Suppl. 91(441): 48-55.

154. **Farrell DJ, Shackcloth J, Barbadora KA, Green MD.** 2006. *Streptococcus pyogenes* isolates with high-level macrolide resistance and reduced susceptibility to telithromycin associated with 23S rRNA mutations. Antimicrob. Agents Chemother. 50(2):817-818

155. **Farrow KA, Lyras D, Rood JL.** 2001. Genomic analysis of erythromycin resistance element Tn*5398* from *Clostridium difficile*. Microbiology. 147(pt 10): 2717-2728

156. **Fehervary P, Lauinger-Lorsch E, Hof H, Melchert F, Bauer I, Zieger W.** 2004. Water birth: microbiological colonisation of the newborn neonatal and maternal infection rate in comparison to conventional bed deliveries. Arch. Gynecol. Obstet. 270(1): 6-9.

157. **Felmingham D, Feldman C, Hryniewicz W, Klugman K, Kohno S, Low DE, Mendes C, Rodloff AC.** 2002. Surveillance of resistance in bacteria causing community acquired respiratory infection. Clin. Microbiol. Infect. 8(suppl 2):12-42.

158. **Fieseler L, Quaiser A, Schleper C, Hantschel U.** 2006. Analysis of the first genome fragment from the marine sponge-associated, novel candidtate phylum Poribacteria by environmental genomics. Environ. Microbiol. 8(4): 612-624.

159. **Fine DH, Hammon BF, Loesche WJ.** 1998. Clinical use of antibiotics in dental practice. Int. J. Antimicrob. Agents. 9(4):235-8.

160. **Flannagan SE, DB Clewell.** 1991. Conjugative transfer of Tn*916* in *Enterococcus faecalis*: trans activation of homologous transposons. J. Bacteriol. 173(22):7136-7141.

161. **Flannagan SE, Zitzow LA, Su YA, Clewell DB.** 1994. Nucleotide sequence of the 18-kb conjugative transposon Tn*916* from *Enterococcus faecalis*. Plasmid. 32(3):350-4.

162. **Fluit AC, FJ Schmitz.** 1999. Class 1 integrons, gene cassettes, mobility and epidemiology. Eur. J. Clin. Microbiol. Infect. Dis. 18(11) : 761-770.

163. **Fluit AC, Visser MR, and Schmitz FJ.** 2001. Molecular detection of antimicrobial resistance. Clin. Microbiol. Rev. 14(4): 836-871

164. **Fine JD, RA Eady.** 1999. Tetracycline and epidermolysis buillosa simplex ; a new indication for one of the oldest and most widely used drugs in dermatology ? Arch. Dermatol. 135(8) :981-982.

165. **Firth N, Apisiridej S, Berg T, O'Rouke BA, Curnock S, Dyke KG, Skurray RA.** 2000. Replication of staphylococcal multiresistance plasmids. J. Bacteriol. 182(8):2170-2178

166. **Fish DN, Piscitelli SC, Danziger LH.** 1995. Development of resistance during antimicrobial therapy: a review of antibiotic classes and patient characteristics in 173 studies. Pharmacotherapy. 15(3): 279-291.

167. **Florez C, Garcia-Lopez JL, Martin-Mazuelos E.** 1997. Susceptibilities of 55 strains of *Neisseria meningitidis* isolated in Spain in 1993 and 1994. Chemotherapy. 43(3):168-70.

168. **Fooks LJ and GR Gibson.** 2002. Probiotics as modulators of the gut flora. Br. J. Nutr. 88(suppl 1):S39-49.

169. **Forge A, J Schacht.** 2000 Aminoglycoside antibiotics. Audiol. Neurootol. 5(1):3-22

170. **Fraenkel GJ.** 1998. Penicillin at the beginning. Ann. Diagn. Pathol. 2(6): 422-424.

171. **Francke C, Siezen RJ, Teusink B.** 2005. Reconstructing the metabolic network of a bacterium from its genome. Trends in Microbiol. 13(11): 550-558.

172. **Frost LS, Leplae R, Summers AO, Toussaint A.** 2005. Mobile genetic elements: the agents of open source evolution. Nat. Revs. Microbiol. 3:722-732.

173. **Frye JG, Jesse T, Long F, Rondeau G, Porwolik S, McClelland M, Jackson CR, Englen M, Fedorka-Cray PJ.** 2006. DNA microarray detection of antimicrobial resistance genes in diverse bacteria. Int. J. Antimicrob. Agents. 27: 138-151.

174. **Fung CP, Yeo Sf, Livermore DM.** 1994. Susceptibility of *Moraxella catarrhalis* isolates to beta-lactam antibiotics in relation to beta-lactamase pattern. J. Antimicrob. Chemother. 33(2):215-222.

175. **Furste JP**. 1987. Conjugative transfer of promiscuous plasmid RP4; plasmid specified functions essential for formation of relaxosomes. P 553- 564 in TJ Kelly and R McMacken (ed.) Mechanisms of DNA replication and recombination. Alan R Liss Inc New York NY.

176. **Gabor EM, Alkema WBL, Janssen DB**. 2004 Quantifying the accessibility of the metagenome by random expression cloning techniques. Env. Microbiol. 6(9):879-886.

177. **Gafan GP, Lucas VS, Roberts GJ, Petrie A, Wilson M, Spratt DA.** 2005. Statistical analyses of comple denaturing gradient gel electrophoresis profiles. J. Clin. Microbiol. 43(8):3971-3978.

178. **Garcia-Vallve S, Romeu A, Palau J.** 2000. Horizontal gene transfer in bacterial and archaeal complete genomes. Genome Res. 10(11) : 1719-1725.

179. **Garmory HS, RW Titball.** 2004. ATP-binding cassette transporters are targets for the development of antibacterial vaccines and therapies. Infect. Imm. 72(12):6757-6763.

180. **Garnier F, Taourit S, Glaser P, Courvalin P, Galimand M.** 2000. Charcterisation of transposon of transposon Tn*1549* conferring VanB-type resistance in *Enterococcus* spp. Microbiol. 146: 1481-1489.

181. **Gautier AL, Dubois D, Escande F, Avril JL, trieu-Cout P, Gaillot O.** 2005. Rapid and accurate identification of human isolates of Pasteurella and related species by sequencing the *sodA* gene. J. Clin. Microbiol. 43(5) : 2307-2314.

182. **Gay K, Stephens DS.** 2001. Structure and dissemination of a chromosomal insertion element encoding macrolide efflux in *Streptococcus pneumoniae*. J. Infect. Dis. 184(1):56-65.

183. **Ge B, McDermott PE, White DG, Meng J.** 2005. Role of efflux pumps and topoisomerase mutations in fluoroquinolones resistance in *Camplylobacter jejuni* and *Campylobacter coli*. Antimicrob. Agents Chemother. 49(8): 3347-3354.

184. **Gevers D, Huys G, Swings J.** 2003. In vitro conjugal transfer of tetracycline resistance from Lactobaciullus isolates to other Gram positive bacteria. FEMS Microbiol. Lett. 225: 125-130.

185. **Gfeller Ky, Roth M, Meile L, Teuber M.** 2003. Sequence and genetic organisation of the 19.3-kb erythromycin and dalfopristin resistance plasmid pLME300 from *Lactobacillus fermentum* ROT1. Plasmid. 50(3):190-201.

186. **Gill SR, Fouts DE Archer GL, Mongodin EF, Deboy RT, Ravel J, Paulsen IT, Kolonay JF, Brinkac L, Beanan M, Dodson RJ, Daugherty SC, Madupu R, Angiuoli SV, Durkin AS, Haft DH, Vamathevan J. Khouri H, Utterback T, Lee C, Dimitrov G, Jiang L, Qin H, Wiedman J, Tran K, Kang K Hance IR, Nelson KE, Fraser CM.** 2005. Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin resistant *Staphylococcus aureus* strain and a biofilm producing methicillin resistant *Staphylococcus epidermidis* strain. J. Bacteriol. 187(7): 2426-2438.

187. **Gill SR, Pop M, Deboy RT, Eckburg PB, Turnbaugh PJ, Samuel BS, Gordon JI, Relman DA, Fraser-Liggett CM, Nelson KE.** 2006. Metagenomic analysis of the human distal gut microbiome. Science. 312 : 1355-1359.

188. **Gillespie DE, Brady SF, Betterman AD, Cianciotto NP, Liles MR, Rondon MR, Clardy J, Goodman RM, Handelsman J.** 2002. Isolation of antibiotics turbomycin A and B from a metagenomic library of soil microbial DNA. Appl. Environ. Microbiol. 68(9): 4301-4306.

189. **Giovanetti E, Brenciani A, Lupidi R, Roberts MC, Varaldo PE.** 2003. Presence of the *tet*(O) gene in erythromycin- and tetracycline-resistant strains of *Streptococcus pyogenes* and linkage with either the *mef*(A) or the *erm*(A) gene. Antimicrob. Agents Chemother. 47(9):2844-9.

190. **Giovanetti E, Brenciani A, Vecchi M, Manzin A, Valaldo PE.** 2005. Prophage association on *mef*(A) elements encoding efflux-mediated erythromycin resistance in Streptococcus pyogenes. J. Antimicrob. Chemother. 55(4) : 445-451.

191. **Giraud E, Baucheron S, Cloeckaert A.** 2006. Resistance to fluoroquinolones in Salmonella: emerging mechanisms and resistance prevention stratergies. Microbs Infect. Mar 24.

192. **Goldman RC, Kadam SK.** 1989. Binding of novel macrolide structures to macrolide-lincosamides-streptogramin B-resistant ribosomes inhibits protein synthesis and bacterial growth. Antimicrob. Agents Chemother. 33(7):1058-1066.

193. **Goldman RC, F Scaglione.** 2004. The macrolide-bacterium interaction and its biological basis. Curr. Drug Targets Infect. Disord. 4(3): 241-260.

194. **Goossens H, Ferech M, Vander Stichele R, Elseviers M, ESAC Project Group.** 2005. Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. Lancet. 365(9459):579-587.

195. **Gourque-Jeannot C, Kalmokoff ML, Kheradpir E, Kwan J, Lampi BJ, McAllister M, Brooks SP.** 2006. Dietary fructooligosaccharides alter the cultivable faecal population of rats, but do not stimulate the growth of intestinal bifidobacteria. Can. J. Microbiol. 52(10):924-933.

196. **Grohmann E, Muth G, and Espinosa M.** 2003. Conjugative plasmid transfer in Gram-positive bacteria. Microbiol. Mol. Biol. Rev. 67(2):277-301.

197. **Gryczan T, Grandi G, Hahn J, Grandi R, Dubnau D.** 1980. Conformational alteration of mRNA structure and the posttranscriptional regulation of erythromycin induced drug resistance. Nucleic Acids Res. 8: 6081-6097.

198. **Guardabassi L, Dijkshoorn L, Collard JM, Olsen JE, Dalsgaard A.** 2000. Distribution and in-vivo transfer of tetracycline resistance determinants in clinical and aquatic Acinetobacter strains. J. Med. Microbiol. 49: 929-936.

199. **Guarner F and JR Malagelada**. 2003. Gut flora in health and disease. Lancet. 24:361(9371):1831.

200. **Guay GG, Khan SA, Rothstein DM.** 1993. The *tet*(K) gene of plasmid pT181 of *Staphylococcus aureus* encodes an efflux protein that contains 14 transmembrane helices. Plasmid. 30(2): 163-166.

201. **Guedon G, Bourgoin F, Pebay M, Roussel Y, Colmin C, Simonet JM, Decaris B.** 1995. Characterisation and distribution of two insertion sequences, IS1191 and isoIS981 in *Streptococcus thermophilus*; does intergeneric transfer of insertion sequences occur in lactic acid bacteria co-cultures? Mol. Microbiol. 16(1): 69-78.

202. **Guerin F, Buu-Hoi A, Mainardi JL, Kac G, Colardelle N, Vaupre S, Gutmann L, Podglajen I.** 2000. Outbreak of methicillin resistant *Staphylococcus aureus* with reduceed susceptibility to glycopeptides in a Parisian hosptial. J. Clin. Microbiol. 38(8) : 29-85-2988.

203. **Guerra B, Soto S, Helmuth R, Mendoza MC.** 2002. Characteristaion of a self-transferable plasmid from *Salmonella enterica* Serotype *Typhimurium* clinical isolates carrying two integrons-borne gene cassettes together with virulence and drug resistance genes. Antimicrob. Agents Chemother. 46(9):2977-2981.

204. **Guggenheim M, Shapiro S, Gmur R, Guggenheim B.** 2001. Spatial arrangements and associative behaviour of species in an in vitro oral biofilm model. Appl. Environ. Microbiol. 67(3):1343-1350.

205. **Guillemot D, C Carbon.** 1999. Antibiotic use and pneumococcal resistance to penicillin: the French experience. Clin. Microbiol. Infect. 5(suppl 4):38-42.

206. **Guiney DG, Hasegawa P.** 1992. Transfer of conjugal elements in oral black pigmented *Bacteroides* (*Prevotella*) spp. involves DNA rearrangements. J. Bacteriol. 174(14): 4853-4855.

207. **Gupta A, Vlamakis H, Shoemaker N, Salyers AA.** 2003. A new Bacteroides conjugative transposons that carries an *erm*B gene. Appl. Env. Microbiol. 69(11):6455-6463.

208. **Gustafsson C, Govindarajan S, Minshull J.** 2004. Codon bias and heterologous protein expression. Trends Biotechnol. 22(7):346-353.

209. **Hancock RE, A Bell.** 1988. Antibiotic uptake into Gram negative bacteria. Eur. J. Clin. Microbiol. Infect. Dis. 7(6):713-720.

210. **Handelsman J.** 2004. Metagenomics: Application of genomics to uncultured Microorganisms. Microbiol. Mol. Biol. Revs. 68(4):669-685.

211. **Hauschild T, Luthje P, Schwarz S.** 2005. Staphylococcal tetracycline-MLSB resistance plasmid pSTE2 is the product of an RSA-mediated in vivo recombination. J. Antimicrob. Chemother. 56(2):399-402.

212. **Hausner M, S Wuertz.** 1999. High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. Appl. Environ. Microbiol. 65(8) : 3710-3713.

213. **Hayashi H, Sakamoto M, Benno Y.** 2002. Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. Microbiol. Immunol. 46(8):535-548.

214. **Hayashi H, Sakamoto M, Kitahara M, Benno Y.** 2003. Molecular analysis of fecal microbiota in elderly individuals using 16S rDNA library and T-RFLP. Microbiol. Immunol. 47(8):557-570.

215. **Hayashi H, Takahashi R, Nishi T, Sakamoto M, Benno Y.** 2005. Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota

using 16S rRNA gene libraries and terminal restriction fragment length polymorphisms. J. Med. Microbiol. 54(Pt 11):1093-1101.

216. **Hayman GT, PL Bolen.** 1993. Movement of shuttle plasmids from *Escherichia coli* into yeasts other than *Saccharomyces cerevisiae* using trans-kingdom conjugation. Plasmid. 30(3):251-257.

217. **Heinemann JA, GF Sprague.** 1996. Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeasts. Nature. 340 :205-209.

218. **Henne A, Daniel R, Schmitz RA, Gottschalk G.** 1999. Construction of environmental DNA libraries in *Escherichia coli* and screening for the presence of genes conferring utilisation of 4-hydroxybutyrate. Appl. Environ. Microbiol. 65(9):3901-3907

219. **Heuer C, Hickman RK, Curiale MS, Hillen W, Levy SB.** 1987. Constituative expression of tetracycline resistance mediated by a Tn*10*-like element in *Haemophilus parainfluenzae* results from a mutation in the repressor gene. J. Bacteriol. 169(3): 990-994.

220. **Hillen W, C Berens.** 1994. Mechanisms underlying expression of Tn*10* encoded tetracycline resistance. Ann. Rev. Microbiol. 48:345-369.

221. **Hinerfeld D, G Churchward.** 2001. Specific binding of integrase to the origin of transfer (oriT) of the conjugative transposon Tn*916*. J. Bacteriol. 183(9):2947-2951.

222. **Hirai T, Heymann JA, Shi D, Sarker R, Maloney PC, Subramanian S.** 2003. Structural mode for 12-helix transporters belonging to the major facilitator superfamily J. Bacteriol. 185(5):1712-1718.

223. **Hiramatsu K, Hanaki H, Ino T, Yabuta K, Oguri T, Tenover FC.** 1997. Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility. J. Antimicrob. Chemother. 40(1):135-6.

224. **Hitch G, Pratten J, Taylor PW.** 2004. Isolation of bacteriophages from the oral cavity. Lett. App. Microbiol. 39:215-219.

225. **Hoiby N.** 2000. Ecological antibiotic policy. J. Antimicrob. Chem. 46 (S1):59-62.

226. **Hooper DC.** 2000. Mechanisms of action and resistance of older and newer fluoroquinolones. Clin. Infect. Dis. 31(suppl)2: S24-28.

227. **Hooton TM, Levy SB.** 2001. Antimicrobial resistance: a plan of action for community practice. Am. Fam. Physician. 63(6):1087-98.

228. **Hopwood Da, KF Chater**. 1980. Fresh approaches to antibiotic production. Philos. Trans. Soc. Lond. Biol. Sci. 290(1040): 313-328.

229. **Houndt T and H Ochman.** 2000. Long-term shifts in patterns of antibiotic resistance in enteric bacteria. Appl. Environ. Microbiol. 66(12):5406-5409.

230. **Hu M, Nandi S, Davies C, Nicholas RA.** 2005. High level chromosomally mediated tetracycline resistance in *Neisseria gonorrhoeae* results from a point mutation in the *rpsJ* gene encoding ribosomal protein S10 in combination with the *mtrR* and *penB* resistance determinants. Antimicrob. Agents. Chemother. 49(10):4327-4334.

231. **Huang C-Y, Patel BK, Mah RA, Baresi L.** 1998. *Caldicellulosiruptor owensensis* sp. nov. an anaerobic extremely thermophilic xylanolytic bacterium. Int. J. Syst. Bacteriol. 48 (1): 91-97.

232. **Hugenholtz P, Goebel BM, Pace NR.** 1998. Impact of culture independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180(18):4765-4774.

233. **Hughes VM, Datta N.** 1983. Conjugative plasmids in bacteria of the 'pre-antibiotic' era. Nature. 302(5910):725-6.

234. **Hummel A, Holzapfel WH, Franz CM.** 2006. Characterisation and transfer of antibiotic resistance genes from enterococci isolated from food. Syst. Appl. Microbiol. 21 (Epub).

235. **Izumiya H.** 2005. Characterisation of isolates of *Salmonella enterica* serovar *typhimurium* displaying high-level fluoroquinolones resistance in Japan. J. Clin. Microbiol. 43(10): 5074-5079.

236. **Jain R, Rivera MC, Lake JA.** 1999. Horizontal gene transfer among genomes: the complexity hypothesis. Proc. Natl. Acad. Sci. USA. 96: 3801-3806.

237. **Jeffries CD, HE Rogers.** 1968. Enhancing effect of agar on swarming by Proteus. J. Bacteriol. 95(2) 732-733.

238. **Jernberg C, Sullivan A, Edlund C, Jansson JK**. 2005. Monitoring of antibiotic-induced alterations in the human intestinal microflora and detection of probiotc strains by use of terminal restriction fragment length polymorphism. Appl. Environ. Microbiol. 71(1): 501-506.

239. **Joan Curcio M, KM Derbyshire**. 2003. The outs and ins of tranpostion: from Mu to Kangeroo. Nature. Revs. Mol. Cell Biol. 4: 1-13.

240. **Johnson AP, Uttley AH, Woodford N, George RC. 1990.** Resistance to vancomycin and teicoplanin: an emerging clinical problem. Clin. Microbiol. Rev. 3(3):280-291.

241. **Johnson DM, Sader HS, Fritsche TR, Biedenbach DJ, Jones RN.** 2006. Emergence of multidrug resistant *S. pneumonia*: report from the SENTRY antimicrobial surveillence programme 1999-2003. Diagn. Microbiol. Infect. Dis. 2006, Mar 16.

242. **Johnson RC, WS Reznikoff.** 1983. DNA sequences at the ends of transposon Tn5 required for transposition. Nature. 304 :280-282.

243. **Johnston NJ, De Azavedo JC, Kellner JD, Low DE.** 1998. Prevalence and characterisation of the mechanism of macrolide, lincosamide and streptogramin resistance in isolates of *Streptococcus pneumoniae.* Antimicrob. Agents Chemother. 42(9):2425-2426.

244. **Kamekura M.** 1998. Diversity of extremely halophilic bacteria. Extremophiles. 2(3): 289-295.

245. **Kaper JB and V Sperandio.** 2005: Bacterial cell-to-cell signalling in the gastrointestinal tract. Infect. Immun. 73(6):3197-3209.

246. **Karami N, Nowrouzian F, Adlerberth I, Wold AE.** 2006. Tetracycline resistance in *Escherichia coli* and persistence in the infantile colonic microbiota. Antimicrob. Agents Chemother. 50(1): 156-161.

247. **Kazimierczak KA, Flint HJ, Scott KP.** 2006. Comparative analysis of sequences flanking *tet*(W) resistance genes in multiple species of gut bacteria. Antimicrob. Agents Chemother. 50(8) : 2632-2639.

248. **Kazor CE, Mitchell PM, Lee Am, Stokes LN, Loesche WJ, Dewhirst FE, Paster BJ.** 2003. Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients. J. Clin. Microbiol. 41(2):558-563.

249. **Kehrenberg C, S Schwarz.** 2001. Molecular analysis of tetracycline resistance in *Pasteurella aerogenes.* Antimicrob. Agents Chemother. 45(10):2885-2890.

250. **Keller J, V Perreten.** 2006. Genetic diversity in fluoroquinolones and macrolide-resistant *Campylobacter coli* from pigs. Vet Microbiol. 10:113 (1-2) 103-108.

251. **Khan SA, Nawaz MS, Khan AA, Cerniglia CE.** 2000. Transfer of erythromycin resistance from poultry to human clinical strains of *Staphylococcus aureus*. J. Clin. Microbiol. 38(5):1832-1838.

252. **Kim JM.** 2006. Antibiotic resistance of *Helicobacyter pylori* isolated from Korean patients. Korean J. Gastroenterol. 47(5): 337-349.

253. **Kimura T, Shiina Y, Sawaio T, Yamaguchi A.** 1998. Cystein-scanning mutagenesis around transmembrane segment III of Tn10-encoded metal-tetracycline/H+ antiporter. J. Biol. Chem. 273(9): 5243-5247.

254. **Kirjavainen PV and GR Gibson.** 1999. Healthy gut microflora and allergy: factors influencing development of the microbiota. Ann. Med. 31(4):288-92.

255. **Kjeldgaard M, Nyborg J.** 1992. Refined structure of elongation factor EF-Tu from *Escherichia coli*. J. Mol. Biol. 223(3):721-42.

256. **Klaasen CH, JW Mouton.** 2005. Molecular detection of the macrolide efflux gene; to discriminate or not between *mef*(A) and *mef*(E). Antimicrob. Agents Chemother. 49(4): 1271-1278.

257. **Klare I, Werner G, Witte W.** 2001. Enterococci. Habitats, infections, virulence factors, resistance to antibiotics, transfer of resistance determinants. Contrib. Microbiol 8 :108-122.

258. **Kleessen B.** 2000. Culture based knowledge on biodiversity, development and stability of human gastrointestinal microflora. Microbial Ecol. Health Dis. 12 suppl 2: 53-63.

259. **Kleinberg I.** 2002. A mixed-bacteria ecological approach to understanding the role of the oral bacteria in dental caries causation: an alternative to *Strepococcus mutans* and the specific-plaque hypothesis. Crit. Rev. Oral Biol. Med. 13(2):108-125.

260. **Klijn N, Weerkamp AH, De Vos WM.** 1991. Identification of mesophilic lactic acid bacteria by using polymerase chain reaction amplified variable regions of 16S rRNA and specific DNA probes. Appl. Environ. Microbiol. 57(11) : 3390-3393.

261. **Kolenbrander PE.** 2000. Oral microbial communities: Biofilms, interactions and genetic systems. Ann. Rev. Microbiol. 54:413-437.

262. **Krause M.** 2002. Resistance problems in gastrointestinal infections. Ther. Umsch. 59(1):36-40.

263. **Krsek M, EM Wellington.** 1999. Comparison of different methods for the isolation and purification of total community DNA form soil. J. Microbiol. Methods. 39(1): 1-16.

264. **Krulwich TA, Lewinson O, Padan E, Bibi E.** 2005. Do physiological roles foster persistence of drugs/multidrug-efflux transporters? A case study. Natur. Rev. Microbiol. 3: 566-572.

265. **Kumar A, HP Schweizer.** 2005. Bacterial resistance to antibiotics: active efflux and reduced uptake. Adv. Drug Deliv. Rev. 57(10):1486-1513.

266. **Kummerer K.** 2004. Resistance in the environment. J. Antimicrob. Chemother. 54: 311-320.

267. **Kuramitsu HK, V Trapa.** 1984. Genetic exchange between oral streptococci during mixed growth. J. Gen. Microbiol. 130(10): 2497-2500.

268. **Kurland CG. Canback B, Berg OG.** 2003. Horizontal gene transfer: a critical review. PNAS. 100(17):9658-9662.

269. **Lacroix J-M, Walker CB.** 1995. Detection and incidence of the tetracycline resistance determinant *tet*(M) in the microflora associated with adult periodontitis. J. Periodontol. 66:102-108.

270. **Lacroix J-M, and Walker CB.** 1996. Detection and prevalence of the tetracycline resistance determinant Tet Q in the microbiota associated with adult periodontitis. Oral Microbiol. Immunol. 11:282-288.

271. **Lambs L, Reverend B DL, Kozlowski H, Berthon G.** 1988. Metal ion-tetracycline interactions in biological fluids. Inorg. Chem. 27 : 3001-3012.

272. **Lancashire JF, Terry TD, Blackall PJ, Jennings MP.** 2005. Plasmid-encoded Tet B Tetracycline resistance in *Haemophilus parasuis*. Antmicrob. Agents Chemother. 49(5):1927-1931.

273. **Lancaster H, Ready D, Mullany P, Spratt D, Bedi R, Wilson M.** 2003. Prevalence and identification of tetracycline-resistant oral bacteria in children not receiving antibiotic therapy. FEMS Microbiol. Lett. 228(1):99-104.

274. **Lancaster H, Roberts AP, Bedi R, Wilson M, Mullany P.** 2004. Characterisation of T*n916S*, a Tn*916*-like element containing the tetracycline resistance determinant *tet*(S). J. Bacteriol. 186(13):4395-4398

275. **Lancaster H, Bedi R, Wilson M, Mullany P.** 2005. The maintenance in the oral cavity of children of tetracycline-resistant bacteria and the genes encoding such resistance. J. Antimicrob. Chemother. 56(3): 524-531.

276. **Lane DJ, Pace B, Olsen GJ, Stahl, Sogin ML, Pace NR.**1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. USA 82(20):6955-6959.

277. **Lanka E, BM Wilkins.** 1995. DNA processing reactions in bacterial conjugation. Ann. Rev. Biochem. 64:141-169.

278. **Launay A, Balllard SA, Johnson PD, Grayson ML, Lambert T.** 2006. Transfer of vancomycin resistance transposon Tn*1549* from *Clostridium symbiosum* to *Enterococcus* spp in the gut of gnotobiotic mice. Antimicrob. Agents Chemother. 50(3):1054-1062.

279. **Lawrence JG, H Ochman.** 1997. Amelioration of bacterial genomes: rates of change and exchange. J. Mol. Evol. 44(4): 383-397

280. **LeClercq R, P Courvalin.** 1991. Intrinsic and unusual resistance to macrolide, lincosamide and streptogramin antibiotics in bacteria. Antimicrobial Agents Chemother. 35(7): 1273-1276.

281. **Leclercq R.** 2002. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clin. Infect. Dis. 34(4):482-92.

282. **Lederberg J.** 1986. Forty years of genetic recombination in bacteria. Nature. 324(6098): 627-628.

283. **Lenski RE, Travisano M.** 1991. Dynamics of adaptation and diversification: a 10 000 generation experiment with bacterial populations. Proc. Natl. Acad. Sci. USA. 91: 6808-6814.

284. **Lepine G, Lacroix JM, Walker CB, Progulske-Fox A.** 1993. Sequencing of *tet*(Q) gene isolated from *Bacteroides fragalis* 1126. Antimicrob. Agents Chemother. 37(9):2037-2041.

285. **Levy SB.** 1984. Resistance to the tetracyclines. P 191-240 in IE Bryan (ed.) Antimicrobial drug resistance. Academic Press, Orlando, Florida.

286. **Levy SB, McMurray LM, Barbosa TM, Burdett V, Courvalin P, Hillen W, Roberts MC, Rood JI, Taylor DE.** 1999. Nomenclature for new tetracycline resistance determinants. Antimicrob. Agents Chemother. 43(6):1523-4.

287. **Li X-Z, Livermore DM, Nikaido H.** 1994. Role of efflux pump(s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol and norfloxacin. Antimicrob. Agents Chemother. 38(8): 1732-1741.

288. **Li Y, Ku CY, Xu J, Saxena D.** 2005. Survey of oral microbial diversity using PCR-based denaturing gradient gel electrophoresis. J. Dent. Res. 84(6):559-564.

289. **Licht TR, A Wilcks.** Conjugative gene transfer in the gastrointestinal environment. Adv. Appl. Microbiol. 58: 77-95.

290. **Liles MR, Manske BF, Bintrim SB, Handelsman J, Goodman RM.** 2003. A census of rRNA genes and linked genomic sequences within a soil metagenomic library. Appl. Environ. Microbiol. 69(5): 2684-2691.

291. **Lipsitch M, MH Samore.** 2002. Antimicrobial use and antimicrobial resistance ; a population perspective. Emerg. Infect. Dis. 8(4) :347-354.

292. **Liras P, JF Martin.** 2006. Gene clusters for beta-lactam antibiotics and control of their expression: why have clusters evolved and from where did they originate? Int. Microbiol. 9(1):9-19.

293. **Livermore DM.** 1993. Determinants of the activity of beta-lactamase inhibitor combinations. J. Antimicrob. Chemother. 31(supplA):9-21

294. **Livermore DM.** 1994. Evolution of beta-lactamase inhibitors. Intensive Care Med. 20(suppl 3):10-13

295. **Livermore D.** 2004. Can better prescribing turn the tide of resistance. Nat. Rev. Microbiol. 2(1):73-78

296. **Livermore DM**. 2004. The need for new antibiotics. Clin. Microbiol. Infect. 10(suppl4):1-9

297. **Liu Y-G, Liu H, Chen L, Qiu W, Zhang Q, Wu H, Yang C, Su J, Wang Z, Tian D, Mei M.** 2002. Development of new transformation-competent artificial chromosome vectors and rice genomic libraries for efficient gene cloning. Gene. 282: 247-255.

298. **Llosa M, Gomis-Ruth FX, Coll M, de la Cruz FF**. 2002. Bacterial conjugation: a two-step mechanism for DNA transport. Mol. Microbiol. 45(1):1-8.

299. **Lorenz MG, W Wackernagel.** 1991. High frequency of natural genetic transformation of *Pseudomonas stutzeri* in soil extract supplemented with a carbon/energy and phosphorus source. Appl. Environ. Microbiol. 57 : 1246-1251.

300. **Lorenz MG, Wackernagel W.** 1994. Bacterial gene transfer by natural genetic transformation in the environment. Microbiol. Rev. 58(3):563-602.

301. **Luna VA, Coates P, Eady EA, Cove JH, Nguyen TTH, Roberts MC.** 1999. A variety of Gram positive bacteria carry mobile *mef* genes. J. Antimicrob. Chemother. 44(1) :19-25.

302. **Luo H, Wan K, Wang HH.** 2005. High-frequency conjugation system facilitates biofilm formation and pAMbeta1 transmission by *Lactococcus lactis*. Appl. Environ. Microbiol.71(6):2970-2978.

303. **Luo N, Pereira S, Sahin O, Lin J, Huang S, Michel L, Zhang Q.** 2005. Enhanced in vivo fitness of fluorquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. PNAS. 102(3): 541-546.

304. **Lyras D, JI Rood.** 2000. Transposition of Tn*4451* and Tn*4453* involves a circular intermediate that forms a promoter for the large resolvase, TnpX. Mol. Microbiol. 38(3):588-601.

305. **Lyras D, Adams V, Lucet I, Rood JI.** 2004. The large resolvase TnpX is the only transposon encoded protein required for transposition of the Tn*4451/3* family of integrative mobile elements. Mol. Microbiol. 51(6):1787-17800.

306. **Maas RM, Gotz J, Wohlleben W, Muth G.** 1998. The conjugative plasmid pSG5 from *Streptomyces ghanaensis* DSM 2932 differs in its transfer functions from other Streptomyces rolling circle type plasmids. Microbiol. 144: 2809-2817.

307. **MacGowan AP, Wise R.** 2001. Establishing MIC breakpoints and the interpretation of in vitro susceptibility tests. J. Antimicrob. Chemother. 48 (Suppl 1):17-28.

308. **Machida C, Y Machida.** 1989. Regulation of IS1 transposition by the insA gene product. J. Mol. Biol. 208:567-574.

309. **MacNeil IA, Tiong CL, Minor C, August PR, Grossman TH, Loiacono KA, Lynch BA, Phillips T, Narula S, Sundaramoorthi R, Tyler A, Aldredge T, Long H, Gilman M, Holt D, Osburne MS.** 2001. Expression and isolation of antimicrobial small molecules from soil DNA libraries. J. Mol. Microbiol. Biotechnol. 3(2):301-308.

310. **Madinier I.** 1991. Oral bacterial flora and its pathogenic potential. J. Biol. Buccale. 19(1):3-15.

311. **Mah TF, O'Toole GA.** 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol. 9(1):34-9.

312. **Mahillon J, Chandler M.** 1998. Insertion sequences. Microbiol. Mol. Biol. Rev. 62(3):725-74.

313. **Maibach H.** 1991. Second generation tetrayclines, a dermatologic overview: clinical uses and pharmacology. Cutis. 48(5): 411-417.

314. **Maisnier-Patin S, Berg OG, Lijas L, Andersson DI.** 2002. Compensatory adaptation to the deleterious effect of antibiotic resistance in *Salmonella typhimurium*. Mol. Microbiol. 2: 355-366.

315. **Majewski J, FM Cohan.** 1999. DNA sequence similarity requirements for interspecific recombination on Bacillus. Genetics. 153(4): 1525-1533.

316. **Maniatis T.** 1989. Molecular Cloning. A laboratory manual. CSH Lab N.Y.

317. **Manichanh C, Rigottier-Gois L, Bonnaud E, Gloux K, Pelletier E, Frangeul L, Nalin R, Jarrin C, Chardon P, Marteau P, Roca J, Dore J.** 2006. Reduced diversity of faecal microbiota in Crohn's disease revealed by a metagenomic approach. Gut. 55(2):205-211.

318. **Marsh PD.** 1999. Microbiologic aspects of dental plaque and dental caries. Dent. Clin. North. Am. 43(4): 599-614.

319. **Marsh PD**. 2003. Are dental diseases examples of ecological catastrophes? Microbiol. 149: 279-294.

320. **Marsh PD.** 2003. Plaque as a biofilm: pharmacological principles of drug delivery and action in the sub- and supragingival environment. Oral Dis. 9 (suppl 1) : 16-22.

321. **Marsh PD.** 2004. Dental plaque as a microbial biofilm. Caries Res. 38(3) 204-211.

322. **Martin R, Langa S, Reviriego C, Jiminez E, Marin ML, Xaus J, Fernandez L, Rodriguez JM.** 2003. Human milk is a source of lactic acid bacteria for the infant gut. J. Pediatr. 143(6) :754-758.

323. **Martinez A, Kolvek SJ, Yip CLT, Hopke J, Brown KA, MacNeil IA, Osburne MS.** 2004. Genetically modified bacterial strains and novel bacterial artificial chromosome shuttle vectors for constructing environmental libraries and detecting heterologous natural products in multiple expression hosts. Appl. Environ. Microbiol. 70(4):2452-2463.

324. **Martinez JL, F Baquero.** 2002. Interactions among stratergies associated with bacterial infection : pathogenicicty, epidemicity and antibiotic reistance. Clin. Microbiol. Revs. 15(4) : 647-679.

325. **Mascini EM, MJ Bonten.** 2005. Vancomycin-resistant enterococci; consequences for therapy and infection control. Clin. Microbiol. Infect. 11(suppl 4): 43-56.

326. **McCartney AL.** 2002. Application of molecular biological methods for studying probiotics and the gut flora. Brit. J. Nutrition. 88(suppl 1): S29-37.

327. **McCaig LF, JM Hughes.** 1995. Trends in antimicrobial drug prescribing among office-based physicians in the US. JAMA. 273(3):214-219.

328. **McKay TL, Ko J, Bilalis Y, DiRienzo JM.** 1995. Mobile genetic elements of *Fusobacterium nucleatum*. Plasmid. 33(1) : 15-25.

329. **McOrist AL, Jackson M, Bird AR.** 2002. A comparison of five methods for extraction of bacterial DNA from human faecal samples. J. Microbiol. Methods. 50(2): 131-139.

330. **Melville CM, Scott KP, Mercer DK, and Flint IJ.** 2001. Novel tetracycline resistance gene, *tet*(32), in the Clostridium-related human colonic anaerobe K10 and its transmission in vitro to the human anaerobe *Butytivibrio fibrisolvens*. Antimicrob. Agents Chemother. 45(11):3242-3249.

331. **Melville CM, Scott KP, Mercer DK, Flint HJ.** 2004. The *Butyrivibrio fibrisolvens tet*(W) gene is carried on the novel conjugative transposon Tn*B1230*, which contains duplicated nitroreductase coding sequences. J. Bacteriol. 186(11):3656-3659.

332. **Mercenier A, Slos P, Faelen M, Lecocq JP.** 1988. Plasmid transduction in *Streptococcus thermophilus*. Mol. Gen. Genet. 212 (2): 386-389.

333. **Mercer DK, Scott KP, Bruce-Johnson WA, Glover A, Flint HJ.** 1999. Fate of free DNA and transformation of the oral bacterium *Streptococcus gordonii* DL1 by plasmid DNA in human saliva. Appl. Environ. Microbiol. 65(1):6-10.

334. **Mercer DK, Scott KP, Melville CM, Glover LA, Flint HJ.** 2001. Transformation of an oral bacterium via chromosomal integration of free DNA in the presence of human saliva. FEMS Microbiol. Lett. 200(2):163-7.

335. **Millar MR, Walsh TR, Linton CJ, Zhang S, Leeming JP, Bennett PM, ALSPAC study team**. 2001. Carriage of antibiotic resistant bacteria by healthy children. J. Antimicrob. Chemother. 47 : 605-610.

336. **Miller DN, Bryant JE, Madsen EL, Ghiorse WC.** 1999. Evaluation and optimisation of DNA extraction and purification procedures for soil and sediment samples. Appl. Environ. Microbiol. 65(11): 4715-4724.

337. **Molin S, Tolker-Nielsen T.** 2003. Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. Curr. Opin. Biotechnol. 14(3):255-61.

338. **Montanari MP, Cochetti I, Mingoia M, Varaldo PE.** 2003. Phenotypic and molecular characterization of tetracycline- and erythromycin-resistant strains of *Streptococcus pneumoniae*. Antimicrob. Agents Chemother. 47(7):2236-41.

339. **Moore PCL, JA Lindsay.** 2002. Molecular Characterisation of the dominant UK methicillin resistant *Staphylococcus aureus* strains: EMRSA-15 and EMRSA-16. J. Med. Microbiol. 51(6): 516-521.

340. **Morhart RE, RJ Fitzgerald.** 1976. Nutritional determinants of the ecology of the oral flora. Dent Clin North Am 20(3): 473-489.

341. **Mori M, Sakagami Y, Ishii Y, Isogai A, Kitada C, Fujino M, Adsit JC, Dunny GM, Suzuki A.** 1988. Structure of cCF10, a peptide sex pheromone which induces conjugative transfer of the *Streptococcus faecalis* tetracycline resistance plasmid, pCF10. J. Biol. Chem. 263(28):14574-14578.

342. **Morris JG Jr, Shay DK, Hebden JN, McCArter RJ, Perdue BE, Jarvis W, Johnson JA, Dowling TC, Polish LB, Schwalbe RS.** 1995. Enterococci resistant to multiple anitmicrobial agents, including vancomycin. Establishment of endemicity in a university medical centre. Ann. Intern. Med. 123(4): 250-259.

343. **Moussard H, Henneke G, Moreira D, Jouffe V, Lopez-Garcia P, Jeanthon C.** 2006. Thermophilic lifestyle for an uncultured archaeon from hydrothermal vents: evidence from environmental genomics. Appl. Environ. Microbiol. 72(3): 2268-2271.

344. **Mueller S, Saunier K, Hanish C, Norin E, Alm L, Midtvedt T, Cresci A, Silvi S, Orpianesi C, Verdenelli MC, Clavel T, Koebnick C, Zunft HJF, Dore J, Blaut M.** 2006. Differences in fecal microbiota in different European Study populations in relation to age, gender, and country: a cross-sectional study. Appl. Environ. Microbiol. 72(2):1027-1033.

345. **Mullany P.** 2000. Gene transfer in the GI tract and the oral cavity. Microbial Ecology in Health and Disease. Suppl 2:73-80.

346. **Mullany P, Roberts AP, Wang H.** 2002. Mechanism of integration and excision in conjugative transposons. Cell Mol. Life Sci. 59:2017-2022.

347. **Munson MA, Banerjee A, Watson TF, Wade WG.** 2004. Molecular analysis of the microflora associated with dental caries. J. Clin. Microbiol. 42(7):3023-3029.

348. **Nakajima Y.** 1999. Mechanisms of bacterial resistance to macrolide antibiotics. J. Infect. Chemother. 5(2) :61-74.

349. **Nazina TN, Tourova TP, Poltaraus AB, Novikova EV, Grigoryan AA, Ivanova AE, Lysenko AM, Petrunyaka VV, Osipov GA, Belyaev SS, Ivanov MV.** 2001. Taxonomic study of aerobic thermophilic bacilli. Int. J. System Evolu. Microbiol.S1:433-446.

350. **Nelson RR.** 1999. Intrinsically resistant gram positive organisms: clinical relevance and implications for infection control. J. Hosp. Infect. 42(4):275-282.

351. **Nesbo CL, Boucher Y, Dlutek M, Doolittle WF.** 2005. Lateral gene transfer ad phylogenetic assignment of environmental fosmid clones. Environ. Microbiol. 7(12) :2011-2026.

352. **Nguyen L, CJ Thompson.** 2006. Foundations of antibiotic resistance in bacterial physiology ; the mycobacterial paradigm. Trends Microbiol. 14(7) :304-312.

353. **Nield BS, Holmes AJ, Gillings MR, Recchia GD, Mabbutt BC, Nevalainen KM, Stokes HW.** 2001. Recovery of new integron classes from environmental DNA. FEMS Microbiol. Lett. 195(1):59-65.

354. **Nielsen HU, Hammerum AM, Ekelund K, Bang D, Pallesen LV, Frimodt-Moller N.** 2004. Tetracycline and macrolide co-resistance in *S. pyogenes*: Co-selection as a reason for increase in macrolide resistance in *S Pyogenes*. Microb. Drug Res. 10(3):231-238.

355. **Nikaido H, DG Thanassi.** 1993. Penetration of lipophilic agents with multiple protonation sites into bacterial cells; tetracyclines and fluoroquinolones as examples. Antimicrob. Agents Chemother. 37:1393-1399.

356. **Nikaido H.** 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. Science. 264:382-386.

357. **Nikaido H.** 1998. Antibiotic resistance casued by Gram negative multidrug efflux pumps. Clin. Infect Dis. 27(suppl.1):S32-41.

358. **Nikolich MP, Shoemaker NB, Salyers AA**. 1992. A Bacteroides tetracycline resistance gene represents a new class of ribosomal protective tetracycline resistance. Antimicrob. Agents Chemother. 36(5):1005-1012.

359. **Nikolich MP, Shoemaker NB, Wang GR, Salyers AA.** 1994. Characterisation of a new type of Bacteroides conjugative transposon TcR emR 7853. J. Bacteriol. 176(21):6606-6612.

360. **Nikolich MP, Hong G, Shoemaker NB, Salyers AA.** 1994. Evidence for natural horizontal transfer of *tet*(Q) between bacteria that normally colonise humans and bacteria that normally colonise livestock. Appl. Environ. Microbiol. 60(9):3255-3260.

361. **Nishikawa M, Suzuki K, Yoshida K.** 1992. DNA integration into recipient yeast chromosomes by trans-kingdom conjugation between *Escherichia coli* and *Saccharomyces cerevisiae*. Curr. Genetics. 21(2) : 101-108.

362. **Nonaka L, Connell SR, Taylor DE.** 2005. 16S rRNA mutations that confer tetracycline resistance in *Helicobacter pylori* decrease drug binding in *Escherichia coli* ribosomes. J. Bacteriol. 187(11):3708-3712.

363. **Norgren M, Scott JR.** 1991. The presence of conjugative transposon Tn*916* in the recipient strain does not impede transfer of a second copy of the element. J. Bacteriol. 173(1):319-24.

364. **Ochman H, Lawrence JG, Groisman EA.** 2000. Lateral gene transfer and the nature of bacterial innovation. Nature. 405 : 299-304.

365. **Ojo KK, Ulep C, Van Kirk N, Luis H, Bernado M, Leitao J, Roberts MC.** 2004. The mef(A) gene predominates among seven macrolide resistance genes identified in Gram negative strains representing 13 genera isolated from healthy Portuguese children. Antimicrob. Agents Chemother. 48(9) : 3451-3456.

366. **Okamoto M, Takano K, Maeda N.** 2001. Distribution of the tetracycline resistance determinant *tet*(O) in black pigmented anaerobes in Japan. Oral Microbiol. Immunol. 16(4): 224-228.

367. **Okitsu N, Kaida S, Yano H, Nakano R, Hosaka Y, Okamoto R, Kobayashi T, Inoue M.** 2005. Characterisation of ermB gene transposition by Tn*1545* and Tn*917* in macrolide resistant *Streptococcus pneumoniae* isolates. J. Clin. Microbiol. 43(1):168-173.

368. **Olsvik B, Olsen I, Tenover FC.** 1994. The *tet*(Q) gene in bacteria isolated from patients with refractory periodontal disease. Oral. Microbiol. Immunol. 10:87-92.

369. **Olsvik B, Olsen I, Tenover FC.** 1995. Detection of *tet*(M) and *tet*(O) using the polymerase chain reaction in bacteria isolated from patients with periodontal disease. Oral Microbiol. Immunol. 10:87-92.

370. **Orth PD, Schnappinger D, Hillen W, Saenger W, Hinrichs W.** 2000. Structural basis of gene regulation by the tetracycline inducible Tet repressor operator system Nat. Struct. Biol. 7:215-219.

371. **Osborn Am, D Boltner.** 2002. When phages, plasmids and transposons collide: genomic islands and conjugative- and mobilisable transposons as a mosaic consortium. Plasmid. 48(3): 202-212.

372. **Osoegawa K, Woon PY, Zhao B, Frengen E, Tateno M, Catanese JJ, de Jong PJ.** 1998. An improved approach for construction of bacterial artificial chromosome libraries. Genomics. 52:1-8.

373. **Osterblad M, Hakanen A, Manninen R, Leistevuo T, Peltonen R, Meurman O, Huovinen P, Kotilainen P.** 2000. A between-species comparison of antimicrobial resistance in enterobacteria in faecal flora. Antimicrob. Agents Chemother. 44(6):1479-1484.

374. **O'Sullivan DJ.** 2000. Methods for analysis of the intestinal microflora. Curr. Issues Intest. Microbiol. 1(2):39-50.

375. **Ott SJ, Musfeldt M, Wenderoth DF, Hampe J, Brant O, Folsch UR, Timmis KN, Schreiber S.** 2003. Reduction in diversity of the colonic mucosa associated bacterial microflora in patients with active inflammatory bowel disease. Gut. 53(2004):685-693.

376. **Ounissi H, P Courvalin**. 2004. Genes required for intrinsic multidrug resistance in *Mycobacterium avium*. Antimicrob. Agents Chemother. 48(9) :3412-3418.

377. **Ouwehand A, Isolauri E, Salminen S.** 2002. The role of the intestinal microflora for the development of the immune system in early childhood. Eur. J. Nutr. 41 suppl. 1:I32-37.

378. **Page WJ, HL Sadoff.** 1976. Control of transformation competence in *Azotobacter vinelandii* by nitrogen catabolite repression. J. Bacteriol. 125:1088-1095.

379. **Patrick DM, Marra F, Hutchinson J, Monnet DL, Ng H, Bowie WR.** 2004. Per capita antibiotic consumption: how does a North American juristiction compare with Europe? Clin. Infect. Dis. 39(1):11-7.

380. **Park HK, Shim SS, Kim SY, Park JH, Park SE, Kim HJ, Kang BC, Kim CM.** 2005. Molecular analysis of colonized bacteria in a human infant gut. J. Microbiol. 43(4) :345-353.

381. **Parr TR, Moore RA, Moore LV, Hancock REW.** 1987. Role of porins in intrinsic antibiotic resistance of *Pseudomonas cepacia.* Antimicrob. Agents Chemother. 31(1):121-123.

382. **Parracho HM, Bingham MO, Gibson GR, McCartney AL.** 2005. Differences between the gut microflora of children with autistic spectrum disorders and that of healthy children. J. Med. Microbiol. 54(part 10):987-991.

383. **Partridge SR, Recchia GD, Scaramuzzi C, Collis CM, Stokes HW and Hall RM.** 2000. Definition of the *attI1* site of class 1 integrons. Microbiology. 146:2855–2864.

384. **Pasquali F, Kehrenberg C, Manfreda G, Schwarz S.** 2005. Physical linkage of Tn*3* and part of Tn*1721* in a tetracycline and ampicicllin resistance plasmid from *Salmonella typhimurium.* J. Antimicrob. Chemother. 55(4):562-565.

385. **Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE.** 2001. Bacterial diversity in human subgingival plaque. J. Bacteriol. 183(12):3770-83.

386. **Patterson AJ, Colangeli R, Spigaglia P, Scott KP.** 2006. Macroarray analysis of the tetracycline and erythromycin resistance genes present in the soil and animal faeces microbiota of six European countries. Environmental Microbiology. Ahead of publication.

387. **Paulsen IT, Brown MH, Skurray RA.** 1996. Proton-dependent multidrug efflux systems. Microbiol. Rev. 60(4):575-608.

388. **Petridis M, Bagdasarian M, Waldor MK, Walker E.** 2006. Horizontal transfer of shiga toxin and antibiotic resistance genes among *E. coli* strains inhouse fly (*Diptera Muscidae*) gut. J. Med. Entomol. 43(2):288-295.

389. **Perea EJ.** 2004. Oral flora in the age of molecular biology. Med. Oral Pathol. Oral Cir. Bucal. 9 suppl 6-10:1-5.

390. **Pereira MS, Barreto VP, Siqueira-junior JP.** 1997. Phage mediated transfer of tetracycline resistance in Staphylococcus aureus isolated form cattle in Brazil. Microbios. 92(372-373) :147-155.

391. **Pérez-Trallero E, Vicente D, Montes M, Marimon JM, Pineiro L.** 2001. High proportion of pharyngeal carriers of commensal streptococci resistant to erythromycin in Spanish adults. J. Antimicrob. Chemother. 48: 225-229.

392. **Pericone CD, Overweg K, Hermans PW, Weiser JN.** 2000. Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. Infect. Imm. 68(7): 3990-3997.

393. **Perreten V, Schwaz F, Cresta L, Boeglin M, Dasen G, Teuber M.** 1997. Antibiotic resistance spread in food. Nature. 389(6653) : 801-802.

394. **Philippe H, CJ Douadyy.** 2003. Horizontal gene transfer and phylogenetics. Curr. Op. Microbiol. 6:498-505.

395. **Pillai SP, Pillai CA, Shankel DM, Mitscher LA.** 2001. The ability of certain antimutagenic agents to prevent development of antibiotic resistance. Mutat. Res. 496(1-2): 61-73.

396. **Pioletti M, Schlunzen F, Harms J, Zarivach R, Gluhmann M, Avila H, Bashan A, Bartels H, Auerbach T, Jacobi C, Hartsch T, Yonath A, Franceschi F.** 2001. Crystal structures of complexes of the small ribosomal subunit with tetracycline, edeine and IF3. EMBO. 20(8):1829-1834.

397. **Ploy MC, Lambert T, Couty JP, Denis F.** 2000. Integrons: an antibiotic resistance gene capture and expression system. Clin. Chem. Lab. Med. 38(6): 483-487.

398. **Poehlsgaard J, S Douthwaite.** 2002. The macrolide binding site on the bacterial ribosome. Curr. Drug Targets Infect. Disord. 2(1):67-78.

399. **Powledge TM.** 2004. New antibiotics – resistance is futile. PloS Biol. 2(2): 53

400. **Poyart C, Berche P, Trieu-Cout P.** 1995. Characterisation of superoxide dismutase genes from Gram positive bacteria by polymerase chain reaction using degenerate primers. FEMS Microbiol. Lett. 131 : 41-45.

401. **Poyart C, Celli J, Trieu-Cout P.** 1995. Conjugative transposition of Tn*916*-related elements from *Enterococcus faecalis* to *Escherichia coli* and *Pseudomonas flurescens*. Antimicrob. Agents Chemother. 39(2):500-506.

402. **Poyart C, Quesne G, Acar P, Berche P, Trieu-Cuot P.** 2000. Characterisation of the Tn*916*-like transposon Tn*3872* in a strain of *Abiotrophia defectiva (Streptococcus defectives)* causing sequential episodes of endocarditis in a child. Antimicrob. Agents Chemother. 44(3):790-793.

403. **Pradier C, Dunais B, Carsenti-Etesse H, Dellamonica P.** 1997. Pneumococcal resistance patterns in Europe. Eur. J. Clin. Microbiol. Infect. Dis. 16(9):644-7.

404. **Prescott, Harley Klein.** 2004. Microbiology. Published by MaGraw Hill Higher Education.

405. **Putman M, Van Veen HW, Konings WN.** 2000. Molecular properties of bacterial multidrug transporters. Microbiol. Mol. Biol. Rev. 64(4): 672-693.

406. **Qian W, Jia Y, Ren SX, He YQ, Feng JX, Lu LF, Sun Q, Ying G, Tang DJ, Wu W, Hao P, Wang L, Jiang BL, Zeng S, Gu WY, Lu G, Rong L, Tian Y, Yao Z, Fu G, Chen B, Fang R, Qiang B, Chen Z, Zhao GP, Tang JL, He C.** 2005. Comparative and functional genomic analyses of the pathogenicity of phytopathogen *Xanthomonas campestris Pv. Campestis*. Genome Res. 15: 757-767.

407. **Quaiser A, Ochsenreiter T, Klenk HP, Kletzin A, Treusch AH, Meurer G, Eck J, Sensen CW, Schleper C.** 2002. First insight into the genome of an uncultivated crenarchaeote from soil. Environ. Microbiol. 4(10): 603-611.

408. **Raadstroem P.** 1991. Recombinational processes involved in the aquisition of sulphonamide resistance in Gram negative bacteria. Acta. Pharma. Nordica. 3(4) :255-256.

409. **Rahal JJ, Urban C, Horn D, Freeman K, Segal-maurer S, Maurer J, Mariano N, Marks S, Burns JM, Dominick D, Lim M.** 1998. Class restriction of cephalosporin use to control total cephalosporin resistance in nosocomial Klebsiella. JAMA. 280(14) : 1233-1237.

410. **Rasiah IA, Wong L, Anderson SA, Sissons CH.** 2005. Variation in bacterial DGGE patterns from human saliva: over time, between individuals and in corresponding dental plaque microcosms. Arch. Oral Biol. 50(9): 779-787.

411. **Rastall RA.** 2004. Bacteria in the gut: friends and foes and how to alter the balance. J. Nutr. 134(8 suppl): S2022-2026.

412. **Ready D, Roberts AP, Pratten J, Spratt DA, Wilson M, Mullany P.** 2002. Composition and antibiotic resistance profile of microcosm dental plaques before and after exposure to tetracycline. J. Antimicrob. Chemother. 49(5):769-75.

413. **Ready D, Bedi R, Spratt DA, Mullany P, Wilson M.** 2003. Prevalence, proportions and identities of antibiotic-resistant bacteria in the oral microflora of healthy children. Microb. Drug Resist. 9(4): 367-372.

414. **Recchia GD, Hall RM.** 1997. Origins of the mobile gene cassettes found in integrons. Trends Microbiol. 5(10):389-94.

415. **Reynolds E, Ross JI, Cove JH.** 2003. Msr(A) and related macrolide/streptogramin resistance determinants ; incomplete transporters ? Int. J. Antimicrob. Agents. 22(3) : 228-236.

416. **Rhee J-K, Ahn DG, Kim YG, Oh JW.** 2005. New thermophilic and therostable esterase with sequence similarity to the hormone-sensitive lipase family, cloned from a metagenomic library. Appl. Environ. Microbiol. 71(2): 817-825.

417. **Ribera A, Roca I, Ruiz J, Gilbert I, Vila J.** 2003. Partial characterisation of a transposon containing the *tet*(A) determinant in a clinical isolate of *Acinetobacter baumannii*. J. Antimicrob. Chemother. 52:477-480.

418. **Rice LB, LL Carias** 1998. Transfer of Tn*5385*, a composite, multiresistance chromosomal element from *Enterococcus faecalis*. J. Bacteriol. 180(3): 714-721.

419. **Rice LB**. 1998. Tn*916* family conjugative transposons and dissemination of antimicrobial resistance determinants. Antimicrob. Agents Chemother. 42(8):1871-1877.

420. **Rice LB.** 2000. Bacterial monopolists: the bundling and dissemination of antimicrobial resistance genes in Gram positive bacteria. Clin. Infect. Dis. 31 : 762-769.

421. **Rice LB.** 2002. Association of different mobile elements to generate novel integrative elements. Cell Mol. Life Sci. 59(12):2023-32.

422. **Rice LB,** Hutton-Thomas R, Lakticova V, Helfand MS, Donskey CJ. 2004. Beta lactams antibiotics and gastrointestinal colonisation with vancomycin resistant enterococci. J. Infect. Dis. 189:1113-1118.

423. **Ridenhour MB, Zellmer DL, Ridenhour RL, Dean RD.** 1996. A novel tetracycline-resistant determinant, *tet*(U), is encoded on the plasmid pKq10 in *Enterococcus faecium*. Plasmid. 35(2):71-80.

424. **Riesenfeld CS, Goodman RM, Handelsman J.** 2004. Uncultured soil bacteria are a reservoir of new antibiotic resistance genes. Environ. Microbiol. 6(9): 981-989.

425. **Roberts AP, Cheah G, Ready D, Pratten J, Wilson M, Mullany P.** 1999. Transfer of a conjugative transposon, Tn*5397* in a model oral biofilm. FEMS Microbiol. Lett. 177(1):63-6.

426. **Roberts AP, Johanesen PA, Lyras D, Mullany P, Rood JI.** 2001. Comparison of Tn5397 from *Clostridium difficile*, Tn*916* from *Enterococcus faecalis* and CW459tet(M) element from *Clostridium perfringens* shows that they

have similar conjugation regions but different insertion and excision modules. Microbiol. 147:1243-1251.

427. **Roberts AP, Cheah G, Ready D, Pratten J, Wilson M, Mullany P.** 2001b. Transfer of Tn*916*-like elements in microsm dental plaques. Antimicrob. Agents Chemother. 45(10):2943-2946.

428. **Roberts MC, Moncla BJ, Hillier SL.** 1991. Characterisation of unusual tetracycline resistant Gram positive bacteria. Antimicrob. Agents Chemother. 35(12): 2655-2657.

429. **Roberts MC.** 1996. Tetracycline-resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. FEMS Microbiol. Rev. 19:1–24.

430. **Roberts MC.** 1998. Antibiotic resistance in oral/respiratory bacteria. Crit. Rev. Oral Biol. Med. 9:522-540.

431. **Roberts MC**.1998. Antibiotic resistance mechanisms in bacteria of oral and upper respiratory origin. Antimicrob. Agents Chemother. 9(1998):255-267.

432. **Roberts MC, Sutcliffe J, Courvalin P, Jensen LB, Rood J, Seppala H.** 1999b. Nomenclature for macrolide and macrolide-lincosamide-streptogramin B resistance determinants. Antimicrob. Agents Chemother. 43(12):2823-2830.

433. **Roberts MC.** 2002. Resistance to tetracycline, macrolide-lincosamide-streptogramin, trimethoprim and sulphonamide drug classes. Mol. Biotechnol. 20(3):261-283.

434. **Roberts MC.** 2004. Resistance to macrolide, lincosamide, streptogramin, ketolide and oxazolidinone antibiotics. Mol. Biotechnol. 28(1):47-62.

435. **Roberts MC**. 2005. Update on acquired tetracycline resistance genes. FEMS Microbiol. Lett. 245: 195-203.

436. **Rodriguez-valera F.** 2004. Environmental genomics ; the big picture ? FEMS Microbiol. Lett 231(2) :153-158.

437. **Rohwer F, R Edwards.** 2002. The phage proteomic tree: a genome based taxonomy for phage. J. Bacteriol. 184(16): 4529-4535.

438. **Rondon M, Raffel SJ, Goodman RM, Handelsman J.** 1999. Towards functional genomics in bacteria: analysis of gene expression in *Escherichia coli* from a bacterial artificial chromosome library of *Bacillus cereus*. Microbiology. 96(11):6451-6455.

439. **Rondon M, August PR, Bettermann AD, Brady SF, Grossman TH, Liles MR, Loiacono KA, Lynch BA, MacNeil IA, Minor C, Tiong CL, Gilman M, Osburne MS, Clardy J, Handelsman J, Goodman RM.** 2000. Cloning the soil metagenome: a stratergy for accessing the genetic and functional diversity of uncultured microorganisms. Appl. Environ. Microbiol. 66(6): 2541-2547.

440. **Rossi-Fedele G, Scott W, Spratt D, Gulabivala K, Roberts AP.** 2006. Incidence and behaviour of Tn*916*-like elements within tetraycline-resistant bacteria isolated from root canals. Oral Microbiol. Immunol. 21(4); 218-222.

441. **Rottem S.** 2003. Interaction of Mycoplasmas with host cells. Physiol. Rev. 83:417-432.

442. **Rowe-Magnus DA, D Mazel.** 2002. The role of integrons in antibiotic resistance gene capture. Int. J. Med. Microbiol. 292(2):115-125.

443. **Saito D, Leonardo rde T, Rodrigues JL, Tsai SM, Hofling JF, Goncalves RB.** 2006. Identification of bacteria in endodontic infections by sequence analysis of 16S rDNA clone libraries. J. Med. Microbiol. 55(Pt 1):101-107.

444. **Sakamoto M, Umeda M, Ishikawa I, Benno Y.** 2000. Comparison of the oral bacterial flora in saliva from a healthy subject and two periodontitis patients by sequence analysis of 16S rDNA libraries. Microbiol. Immunol. 44(8):643-652.

445. **Sakamoto M, Takeuchi Y, Umeda M, Ishikawa I, Benno Y.** 2003. Application of terminal RFLP analysis to characterise oral bacterial flora in saliva of healthy subjects and patients with periodontitis. J. Med. Microbiol. 52: 79-89.

446. **Sakamoto M, Huang Y, Osnishi M, Umeda M, Ishikawa I, Benno Y.** 2004. Changes in oral microbial profiles after periodontal treatment as determined by molecular analysis of 16S rRNA genes. J. Med. Microbiol. 53:563-571.

447. **Sakamoto M, Umeda M, Benno Y.** 2005. Molecular analysis of human oral microbiota. J. Periodontal Res. 40(3):277-285.

448. **Salyers AA, NB Shoemaker.** 1992. Chromosomal gene transfer elements of the Bacteroides group. Eur. J. Clin. Microbiol. Infect. Dis. 11(11):1032-1038.

449. **Salyers AA, Shoemaker NB, Stevens AM, and Li LY.** 1995. Conjugative transposons: an unusual and diverse set of integrated gene transfer elements. Microbiol. Rev. 59(4):579-590.

450. **Salyers AA, Amabile-Cuevas CF.** 1997. Why are antibiotic resistance genes so resistant to elimination? Antimicrob. Agents Chemother. 41(11):2321-5.

451. **Salyers AA, Gupta A, Wang Y.** 2004: Human intestinal bacteria a reservoir for antibiotic resistance genes. Trends Microbiol. 12(9):412-416

452. **Sanai Y, Persson GR, Starr JR, Luis HS, Bernardo M, Leitao J, Roberts MC.** 2002. Presence and antibiotic resistance of *Porphyromonas gingivalis, Prevotella intermedia,* and *Prevotella nigrescens* in children. J. Clin. Periodontol. 29(10):929-934.

453. **Sanchez-Pescador R, Brown JT, Roberts M, Urdea MS.** 1988. Homolgy of the Tet(M) with translational elongation factors: implications for potential modes of *tet*M conferred tetracyline resistance. Nucleic Acids. Res. 16(3): 1218.

454. **Schloss PD, J Handelsman.** 2003. Biotechnological prospects from metagenomics. Curr. Op. Biotechnol. 14: 303-310.

455. **Schloss PD, J Handelsman.** 2004. Status of the microbial census. Microbiol. Mol. Biol. Revs. 68(4):686-691.

456. **Schnappinger D, W Hillen.** 1996. Tetracyclines; antibiotic action, uptake and resistance mechanisms. Arch. Microbiol. 165(6): 359-369.

457. **Schneierson SS.** 1948. Serological and biological characteristics and penicillin resistance of non-haemolytic streptococci isolated from subacute bacterial endocarditis. J. Bacteriol. 55(3):393-399.

458. **Schrag SJ, Perrot V, Levin BR.** 1997. Adaptation to the fitness costs of antibiotic resistance in *Escherichia coli*. Proc. Biol. Sci. 264(1386):1287-1291.

459. **Schwarz S, Kehrenberg C, Ojo KK.** 2002. *Staphylococcus sciuri* gene *erm*(33), encoding inducible resistance to macrolides, lincosamides and streptogramin B antibiotics is a product of recombination between *erm*(C) and *erm*(A). Antimicrob. Agents. Chemother. 46(11):3621-3623.

460. **Scott GW.** 1949. Spontaneous mutation to streptomycin resistance in *Escherichia coli*. Br. J. Exp. Pathol. 30(5): 501-505.

461. **Scott JR, GG Churchward.** Conjugative transpsoition. Ann. Rev. Microbiol. 49:367-397

462. **Scott KP.** 2002. The role of conjugative transposons in spreading antibiotic resistance between bacteria that inhabit the gastrointestinal tract. Cell. Mol. Life Sci. 59(12):2071-2082.

463. **Scott KP, Melville CM, Barbosa TM, Flint HJ.** 2000. Occurrence of the new tetracycline resistance gene *tet*(W) in bacteria from the human gut. Antimicrob. Agents Chemother. 44:775-777.

464. **Sebaihia M, Wren BW, Mullany P, Fairweather NF, Minton N, Stabler R, Thomson NR, Roberts AP, Cerdeno-Tarraga Am, Wang H, Holden MT, Wright A, Churcher C, Quail MA, Baker S, Bason N, Brooks K, Chillingworth T, Cronin A, Davis P, Dowd L, Fraser A, Feltwell T, Hance Z, Holroyd S, Jagels K, Oule S, Mungall K, Price C, Rabbinowitsch E, Sharp S, Simmonds M, Stevens K, Unwin L, Whithead S, Dupay B, Dougan G, Barrell B, Parkhill J.** 2006. The multidrug resistant human pathogen *Clostridium difficile* has a highly mobile, mosaic genome. Nat. Genet. 38(7):779-786.

465. **Sebat JL, Colwell FS, Crawford RL.** 2003. Metagenomic profiling: Microarray analysis of an environmental genomic library. Appl. Env. Microbiol. 69(8):4927-4934.

466. **Seguritan V, Feng IW, Rohwer F, Swift M, Segall AM.** 2003. Genome sequence of two closely related *Vibrio parahaemolyticus* phages VP16T and VP16C. J. Bacteriol. 185(21):6434-6447.

467. **Sekiguchi J, Asaqi T, Miyoshi-Akiyama T, Fujino T, Kobayashi I, Morita K, Kikuchi Y, Kuratsuji T, Kirikae T.** 2005. Multidrug resistant *Pseudomonas aeruginosa* strain that caused an outbreak in a neurosurgery ward and its acc(6')-Iae gene casseyte encoding a novel aminoglycoside acetyltransferase. Antimicrob. Agents Chemother. 49(9): 3734-3742.

468. **Senghas E, Jones JM, Yamamoto M, Gawron-Burke C, Clewell DB.** 1988. Genetic organization of the bacterial conjugative transposon Tn*916*. J. Bacteriol. 170(1):245-9.

469. **Seral C, Castillo FJ, Garcia C, Rubio-Calvo MC, Gomez-Lus R.** 2000. Presence of conjugative transposon Tn*1545* in strains of *Streptococcus pneumoniae* with *mef*(A), *erm*(B), *tet*(M), *catpC194* and *aph3'III* genes. Enfirm. Infect. Microbiol. Clin. 18(10): 506.

470. **Shapiro JA.** 1992. Natural genetic engineering in evolution. Genetica. 86(1-3) : 99-111.

471. **Shi HN, A Walker.** 2004. Bacterial colonisation and the development of intestinal defences. Can. J. Gastroenterol. 18(8):493-500.

472. **Shimizu K, Ogura H, Goto M, Ashara T, Nomoto K, Morotomi M, Yoshiya K, Matsushima A, Sumi Y, Kuwagata Y, Tanaka H, Shimazu T, Sugimoto H.** 2006. Altered gut flora and environment in patients with severe SIRS. J. Trauma. 60(1): 126-133.

473. **Shizuya H, Birren B, Kim UJ, Mancino V, Slepak T, Tachiiri Y, Simon M.** 1992. Cloning and stable maintainance of 300 kilobase pair fragments of

human DNA in *Escherichia coli* using an F-factor based vector. Proc. Natl. Acad. Sci. USA. 89:8794-8797.

474. **Shizuya H, H Kouros-Mehr.** 2001. The development and applications of the bacterial artificial chromosome cloning system. Keio. J. Med. 50(1): 26-30.

475. **Shoemaker NB, Guthrie EP, Salyers AA, Gardner JF.** 1985. Evidence that the clindamycin erythromycin resistance gene of Bacteroides plasmid pBF4 is on a transposable element. J. Bacteriol. 162(2):626-632.

476. **Shoemaker NB, Anderson KL, Smithson Sl, Wang GR, Salyers AA.** 1991. Conjugal transfer of a shuttle vector from the human colonic anaerobe *Bacteroides uniformis* to the ruminal anaerobe *Prevotella (Bacteroides) ruminicola* B14. Appl. Environ. Microbiol. 57(8):2114-2120.

477. **Shoemaker NB, Wang GR, Slayers AA.** 1992. Evidence for natural transfer of a tetracycline resistance gene between bacteria from the human colon and bacteria from the bovine rumen. Appl. Environ. Microbiol. 58(4):1313-1320.

478. **Shoemaker NB, Vlamakis H, Hayes K, Salyers AA.** 2001. Evidence for extensive resistance gene transfer among *Bacteroides* spp and among *Bacteroides* and other genera in the human colon. Appl. Environ. Microbiol. 67(2):561-568.

479. **Shultz TR, Tapsall JW, White PA, Ryan CS, Lyras D, Rood JI, Binotto E, Richardson CJ.** 2003. Chloramphenicol resistant *Neisseria meningitides* containing *catP* isolated in Australia. J. Antimicrob. Chemother. 52(5):856-859.

480. **Simjee S, MJ Gill.** 1997. Gene transfer, gentamicin resistance and enterococci. J. Hosp. Infect. 36(4): 249-259.

481. **Sinclaire W, Jordaan HF, Global alliance to improve outcomes in acne.** 2005. Acne guideline 2005 update. S. Afr. Med. J. 95(11 pt 2): 881-892.

482. **Smith CJ, FL Macrina.** 1984. Large transmissible clindamycin resistance plasmid in *Bacteroides ovatus*. J. Bacteriol. 158(2):739-741.

483. **Smith DL, Dushoff J, Morris G.** 2005. Agricultural antibiotics and human Health. PloS Medicine. 2(8): 731-735.

484. **Solomon JM, AD Grossman** 1996. Who's competent and when: regulation of natural genetic competence in bacteria. Trends Genet. 12(4) : 150-155.

485. **Sosio M, Giusino F, Cappellano C, Bossi E, Puglia Am, Donadio S.** 2000. Artificial chromosomes for antibiotic-producing actinomycetes. Nat. Biotechnol. 18(3):343-345.

486. **Sosio M, Bossi E, Donadio S.** 2001. Assembly of large genomic segments in artificial chromosomes by homologous recombination in *Escherichia coli*. Nucl. Acids Res. 29(7):37.

487. **Sougakoff W, Papadopoulou B, Nordmann P, Courvalin P.** 1987. Nucleotide sequence and distribution of gene *tet*(O) encoding tetracycline resistance in *Campylobacter coli*. FEMS Microbiol. Lett. 44:153-159.

488. **Spahn CMT, Blaha G, Agrawai RK, Penczek P, Grassucci RA, Trieber CA, Connell SR, Taylor DE, Nierhaus KH, Frank J.** 2001. Localisation of the ribosomal protection protein *tet*(O) on the ribosome and mechanism of tetracycline resistance. Mol. Cell. 7:1037-1045.

489. **Speer BS, AA Salyers.** 1989. Novel aerobic tetracycline resistance gene that chemically modifies tetracycline. J. Bacteriol. 171(1):148-153.

490. **Speer BS, Bedzyk L, Salyers AA.** 1991. Evidence that a novel tetracycline resistance gene found on two bacteroides transposons encodes an NADP-requiring oxidoreductase. J. Bacteriol. 173(1):176-83.

491. **Speer BS, Shoemaker NB, Salyers AA.** 1992. Bacterial resistance to tetracycline: mechanisms, transfer and clinical significance. Clin. Microbiol. Rev. 5(4): 387-399.

492. **Spiegelman D, Whissell G, Greer CW.** 2005. A survey of the methods for the characterisation of microbial consortia and communities. Can. J. Microbiol. 51(5): 355-386.

493. **Spiliopoulou I, Petinaki E, Papandreou Dimitracopoulos G.** P2004. *erm*(C) is the predominant genetic determinant for the expression of resistance to

macrolides among methicillin resistant *Staphylococcus aureus* clinical isolates in Greece. J. Antimicrob. Chemother. 53:814-817.

494. **Stanton TB, SB Humphrey.** 2003. Isolation of tetracycline resistant Megasphaera elsdenii strains with novel mosaic gene combinations of *tet*(O) and *tet*(W) from swine. Appl. Environ. Microbiol. 69(7) : 3874-3882.

495. **StantonTB, Scott KP.** 2005. Hybrid *tet* genes and *tet* gene nomenclature : request for opinions. Antimicrob. Agents Chemother. 49(3) :1265-1266.

496. **Stark CA, Edlund C, Sjostedt S, Kristensen G, Nord CE.** 1993. Antimicrobial resistance in human oral and intestinal anaerobic microfloras. Antimicrob. Agents Chemother. 37(8) :1665-1669.

497. **Steele HL, WR Streit.** 2005. Metagenomics: Advances in ecology and biotechnology. FEMS Microbiol. Lett. 247(2):105-111.

498. **Stein JL, Marsh TL, Wu KY, Shizuya H, DeLong EF.** 1996. Characterisation of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genome fragment from a planktonic marine archaeon. J. Bacteriol. 178(3):591-599.

499. **Stephen AM and JH Cummings.** 1980. The microbial contribution to human faecal mass. J. Med. Microbiol. 13(1):45-56.

500. **Stokes HW, Holmes AJ, Nield BS, Holley MP, Helena Nevalainen KM, Mabbutt BC, Gillings MR.** 2001. Gene cassette PCR; sequence-independent recovery of entire genes from environmental DNA. Appl. Environ. Microbiol. 67(11): 5240-5246.

501. **Storrs MJ, Poyart-Salmeron C, Trieu-Cout P, Courvalin P.** 1991. Conjugative transposition of Tn*916* requires the excisive and integrative activities of the transposon-encoded integrase. J. Bacteriol. 173(14):4347-4352.

502. **Stratchounski LS, Taylor EW, Dellinger EP, Pechere JC.** 2005. Antibiotic policies in surgery: a consensus paper. Int. J. Antimicrob. Agents. 26(4): 312-322

503. **Streit WR, RA Schmitz.** 2004. Metagenomics – the key to the uncultured microbes. Curr. Op. Microbiol. 7: 492-498.

504. **Stroman P, Muller CC, Sorensen KI.** 2003. Heat shock treatment increases the frequency of loss of an erythromycin resistance encoding transposable element from the chromosome of *Lactobacillus crispatus* CHCC3692. Appl. Env. Microbiol. 69(12):7173-7180.

505. **Stone BJ, YA Kwaik.** 1999. Natural competence for DNA transformation by *Legionella pneumophila* and its association with expression of type IV pili. J. Bacteriol. 181(5): 1395-1402.

506. **Strong SJ, Ohta Y, Litman GW, Amemiya CT.** 1997. Marked improvement of PAC and BAC cloning is achieved using electroelution of pulsed-field gel-separated partial digests of genomic DNA. Nucl. Acids Res. 25(19):3959-3961.

507. **Su YA, He P, Clewell DB.** 1992. Characterisation of the *tet*(M) determinant of Tn*916*: evidence for regulation by transcription attenuation. Antimicrob. Agents Chemother. 36(4):769-778.

508. **Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, Dore J.** 1999. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl. Env. Microbiol. 65(11):4799-4807.

509. **Sun YH, Exley R, Li Y, Goulding D, Tang C.** 2005. Identification and characterisation of genes required for competence in *Neisseria menigitidis*. J. Bacteriol. 187(9) :3273-3276.

510. **Syed SA, WJ Loesche.** 1972. Survival of human dental plaque flora in various transport media. Appl. Microbiol. 24(4) : 638-644.

511. **Takai K, Moyer CL, Miyazaki M, Nogi Y, Hirayama H, Nealson KH, Horikoshi K.** 2005. *Marinobacter alkaliphilus* sp. nov. a novel alkaliphilic bacterium isolated from subseafloor alkaline serpentine mud from Ocean Drilling Programme Site 1200 at South Chamorro Seamount, Mariana Foreare. Extremophiles. 9(1): 17-27.

512. **Tally FP, Snydman DR, Shimell MJ, Malamy MH.** 1982. Charecterisation of pBFTM10 a clindamycin-erythromycin resistance transfer factor from *Bacteroides fragalis*. J. Bacteriol. 151(2):686-691.

513. **Tan SY.** 2003. Antonie van Leeuwenhoek (1632-1723): Father of microscopy. Singapore Med. J. 44:557-558.

514. **Tankovic J, Leclercq R, Duval J.** 1993. Antimicrobial susceptibility of *Pediococcus* spp. and genetic basis of macrolide resistance in *Pediococcus acidilacticic* HM3020. Antimicrob. Agents Chemother. 37(4):789-792.

515. **Tanner A, Maiden MF, Paster BJ, Dewhirst FE.** 2000. The impact of 16S ribosomal RNA-based phylogeny on the taxonomy of oral bacteria. Periodontol. 5:26-51.

516. **Tannock GW.** 2001. Molecular assessment of intestinal microflora. Am. J. Clin. Nutr. 73(Suppl):410S-414S.

517. **Tannock GW.** 2002. Analysis of the intestinal microflora using molecular methods. Euro. J. Clin. Nutrit. 56(suppl 4): 44-49.

518. **Tatum EL, Lederberg J.** 1947. Gene recombination in the bacterium *Escherichia coli*. J. Bacteriol. 53(6): 673-684.

519. **Taylor DE, A Chau.** 1996. Tetracycline resistance mediated by ribosomal protection. Antimicrob. Agents. Chemother. 40(1):1-5.

520. **Tebbe CC, W Vahjen.** 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. Appl. Environ. Microbiol. 59(8): 2657-2665.

521. **Tenover FC, McGowan JE Jr.** 1996. Reasons for the emergence of antibiotic resistance. Am. J. Med. Sci. 311(1):9-16.

522. **Tenover FC, Weigel LM, Appelbaum PC, McDougal LK, Chaitram J, McAllister S, Clark N, Killgore G, O'Hara CM, Jevitt L, Patel JB, Bozdogan B.** 2004. Vancomycin resistant *Staphylococcus aureus* isolate from a patient in Pensylvania. Antimicrob. Agents Chemother. 48(1):275-280.

523. **Tenson T, A Mankin.** 2006. Antibiotics and the ribosome. Mol. Microbiol. 59(6):1664-1677.

524. **Teuber M, Schwarz F, Perreten V.** 2003. Molecular structure and evolution of the conjugative multiresistance plasmid pRE25 of *Enterococcus faecalis* isolated from a raw-fermented sausage. Int. J. Food Microbiol. 88(2-3):325-329.

525. **Thomas CM, KM Nielsen** 2005. Mechanisms of and barriers to horizontal gene transfer between bacteria. Nat. Rev. Microbiol. 3(9): 711-721.

526. **Thomas WD, GL Archer.** 1989. Mobility of gentamicin resistance genes from staphylococci isolated in the United States: identification of Tn*4031*, a gentamicin resistance transposon from *Staphylococcus epidermidis*. Antimicrob. Agents Chemother. 33(8):1335-1341.

527. **Thompson CJ, Kieser T, Ward JM, Hopwood DA.** 1982. Physical analysis of antibiotic resistance genes from Streptomyces and their use in vector construction. Gene. 20(1): 51-62.

528. **Tjellstrom B, Stenhammer L, Hogberg L, Falth-Magnusson K, Magnusson KE, Midtvedt T, Sundgvist T, Norin E.** 2005. Gut microflora associated characteristics in children with celiac disease. Am. J. Gastroenterol. 100(12): 2784-2788.

529. **Tompkins GR, Peavey MA, Birchmeier KR, Tagg JR.** 1997. Bacteriocin production and sensitivity among coaggregating and noncoaggregating oral streptococci. Oral Microbiol. Immunol. 12(2): 98-105.

530. **Torsvik V, L Overeas.** 2002. Microbial diversity and function in soil: from genes to ecosystems. Curr. Op. Microbiol. 5: 240-245.

531. **Tortoli E.** 2003. Impact of genotypic studies on mycobacterial taxonomy; the new Mycobacteria of the 1990s. Clin. Microbiol. Revs. 16(2) :319-354.

532. **Trallero EP, Fernandez-Mazarrasa C.** 2001. Spanish Surveillance Group for Respiratory Pathogens. 2001. Antimicrobial susceptibilities of 1,684 *Streptococcus pneumoniae* and 2,039 *Streptococcus pyogenes* isolates and their

ecological relationships: results of a 1-year (1998-1999) multicenter surveillance study in Spain. Antimicrob. Agents Chemother. 45(12):3334-40.

533. **Treusch AH, Kletzin A, Raddatz G, Ochsenreiter T, Quaiser A, Meurer G, Schustar SC, Schieper C.** 2004. Characterisation of large insert DNA libraries from soil for environmental genomic studies of Archaea. Environ. Microbiol. 6(9): 970-980.

534. **Trevors JT** 1996. DNA in soil: adsorption, genetic transformation, molecular evolution and genetic microchip. Antonie van Leeuwenhoek. 70(1): 1-10.

535. **Tuohy K, Davies J, Rumsby P, Rumney C, Adams MR, Rowland IR.** 2002. Monitoring transfer of recombinant and non-recombinant plasmids between *L lactis* strains and members of the human gastrointestinal microbiota: in vivo impact of donor cell numbers and diet. J. Appl. Microbiol. 93(6):954-964.

536. **Turnidge J.** 2001. Responsible prescribing for upper respiratory tract infections. Drugs. 61(14):2065-2077.

537. **Twiss E, Coros AM, Tavakoli NP, Derbyshire, KM.** 2005. Transposition is modulated by a diverse set of host factors in *Escherichia coli* and is stimulated by nutritional stress. Mol. Microbiol. 57(6) : 1593-1607.

538. **Tyson GW, JF Banfield.** 2005. Cultivating the uncutivated ; a community genomics perspective. Trends Microbiol. 13(9) : 411-415.

539. **Ubukata K, Konno M, Fujii R.** 1975. Transduction of drug resistance to tetracycline, chloramphenicol, lincomycin and clindamycin with phages induced from *Streptococcus pyogenes*. J. Antibiot. 28(9):681-688.

540. **Upreti RK, Shrivastava R, Charturvedi UC.** 2004. Gut microflora and toxic metals: chromium as a model. Indian. J. Med. Res. 199:49-59.

541. **Vaahtovuo J, Korkeamaki M, Munukka E, Viljanen MK, Toivanen P.** 2005. Quantification of bacteria in human feces using 16S rRNA-hybridisation, DNA-staining and flow cytometry. J. Microbiol. Methods. 63: 276-286.

542. **Vaishnavi C, S Kaur.** 2005. Is Campylobacter involved in antibiotic associated diarrhoea? Indian J. Pathol. Microbiol. 48(4): 526-529.

543. **van Bambeke, Balzi E, Tulkens PM.** 2000. Antibiotic efflux pumps. Biochem. Pharmacol. 60(4): 457-470.

544. **van den Bogaard, Bruinsma N, Stobberingh EF.** 2000. The effect of banning avoparcin on VRE carriage in the Netherlands. J. Antimicrob. Chemother. 46:145-153.

545. **van Veen HW, WN Konings.** 1998. The ABC family of multidrug transporters in microorganisms. Biochim. Biophys. Acta. 1365(1-2):31-36.

546. **Van Wilkelhoff AJ and K Boutaga.** 2005. Transmission of periodontal bacteria and models of infection. J. Clin. Periodontol. 32 suppl. 6:16-27.

547. **Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W, Futs DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers YH, Smith HO.** 2004. Enviromental genome shotgun sequencing of the Sargasso sea. Science. 304(5667): 66-74.

548. **Vergin KL, Urbach E, Stein JL, DeLong EF, Lanoil BD, Giovannoni SJ.** 1998. Screening of a fosmid library of marine environmental genomic DNA fragments reveals four clones related to members of the order Planctomycetales. Appl. Environ. Microbiol. 64(8): 3075-3078.

549. **Versalovic J, Osato MS, Spakovsky K, Pina Dore M, Reddy R, Stone GG, Shortridge D, Flamm RK, Tanaka SK, Graham DY.** 1997. Point mutations in the 23S rRNA gene of *Helicobacter pylori* associated with different levels of clarithromycin resistance. J. Antimicrob. Chemother. 40: 283-286.

550. **Vester B, S Douthwaite.** 2001 Macrolide resistance conferred by base substitutions in 23S rRNA. Antimicrob. Agents Chemother. 45(1):1-12.

551. **Villedieu A, Diaz-Torres ML, Hunt N, McNab R, Spratt DA, Wilson M, Mullany P.** 2003. Prevalence of tetracycline resistance genes in oral bacteria. Antimicrob. Agents Chemother. 47(3):878-882.

552. **Villedieu A, Diaz-Torres ML, Roberts AP, Hunt N, McNab R, Spratt DA, Wilson M, Mullany P.** 2004. Gentic basis of erythromycin resistance in oral bacteria. Antimicrob. Agents Chemother. 48(6): 2298-2301.

553. **Voget S, Leggewie C, Usebeck A, Raasch C, Jaeger KE, Streit WR.** 2003. Prospecting for novel biocatalysts in a soil metagenome. Appl. Environ. Microbiol. 69(10): 6235-6242.

554. **Wade W.** 2002. Unculturable bacteria – the uncharacterized organisms that cause oral infections. J. R. Soc. Med. 95:81-83.

555. **Walker CB.** 2000. The acquisition of antibiotic resistance in the periodontal microflora. Periodontol. 2000 10: 79-88.

556. **Walker ES, Neal CL, Laffan E, Kalbfleisch JH, Berk SL, Levy F.** 2000. Long-term trends in susceptible *M. catarrhalis*: a population analysis. J. Antimicrob. Chemother. 45(2):175-182.

557. **Wang BY, Chi B, Kuramitsu HK.** 2002. Genetic exchange between *Treponema denticola* and *Streptococcus gordonii* in biofilms. Oral Microbiol. Immunol. 17(2):108-12.

558. **Wang GY, Graziani E, Waters B, Pan W, Li X, McDermott J, Meurer G, Saxena G, Andersen RJ, Davies J.** 2000. Novel natural products from soil DNA libraries in a streptomycete host. Org. Lett. 2(16): 2401-2404.

559. **Wang H, Roberts AP, Lyras D, Rood JI, Wilks M, Mullany P.** 2000. Characterisation of the ends and target sites of the novel conjugative transposon Tn*5397* from *Clostridium difficile*: excision and circularisation is mediated by the large resolvase TndX. J. Bacteriol. 182(13):3775-3783.

560. **Wang H, Roberts AP, Mullany P.** 2002 DNA sequence of the insertional hot spot of Tn*916* in the *Clostridium difficile* genome and discovery of a Tn*916*-like

element in an environmental isolate integrated in the same hot spot. FEMS Microbiol. Lett. 192:15-20.

561. **Wang M, Ahrne S, Jeppsson B, Molin G.** 2005. Comparison of bacterial diversity along the human intestinal tract by direct cloning and sequencing of 16S rRNA genes. FEMS Microbiol. Ecol. 54(2):219-231.

562. **Wang RF, Cao WW, Cerniglia CE.** 1996. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl. Env. Microbiol. 62(4):1242-1247.

563. **Wang X, Heazlewood SP, Krause DO, Florin TH.** 2003. Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. J. Appl. Microbiol. 95(3):508-520.

564. **Wang Y, Taylor DE.** 1991. A DNA sequence upstream of the *tet*(O) gene is required for full expression of tetracycline resistance. Antimicrob. Agents Chemother. 35(10) :2020-2025.

565. **Webber M, LJV Piddock.** 2003. The importance of efflux pumps in bacterial resistance. J. Antimicrob. Chemother. 51:9-11.

566. **Wegener HC, Aarestrup FM, Gerner-Smidt P, Bager F.** 1999. Transfer of antibiotic resistant bacteria from animals to man. Acta. Vet Scand. Suppl 92:51-57.

567. **Weinbauer MG, F Rassoulzadegan.** 2004. Are viruses driving microbial diversification and diversity? Environ. Microbiol. 6(1):1-11.

568. **Weinbauer MG**. 2004. Ecology of prokaryotic viruses. FEMS Microbiol. Rev. 28(2):127-181.

569. **Weisblum B.** 1995. Erythromycin resistance by ribosome modification. Antimicrob. Agents Chemother. 39:577-585.

570. **Wenzel SC, R Muller.** 2005. Recent developments towards the heterologous expression of complex bacterial natural product biosynthetic pathways. Curr. Opin. Biotechnol. 16(6): 594-606.

571. **Werckenthin C, Schwarz S, Westh H**. 1999. Structural alterations in the translational attenuator of constitutively expressed *ermC'* genes. Antimicrob. Agents Chemother. 43(7): 1681-1685.

572. **Whittle G, Hund BD, Shoemaker NB, Salyers AA**. 2001. Characterization of the 13-kilobase ermF region of the Bacteroides conjugative transposon CTnDOT. Appl. Environ. Microbiol. 67(8):3488-95.

573. **Wicker T, Guyot R, Yahiaoui N, Keller B.** 2003. CACTA Transposons in Triticeae. A diverse family of high-copy repetitive elements. Plant Physiol. 132: 52-63.

574. **Wiener P, Egan S, Huddleston AS, Wellington EM.** 1998. Evidence for transfer of antibiotic resistance genes in soil populations of streptomycetes. Mol. Ecol. 7(11):1205-1216.

575. **Wilkins JC, Beighton D, Homer KA.** 2003. Effect of acidic pH on expression of surface-asociated proteins of *Streptococcus oralis.* Appl. Env. Microbiol. 69(9):5290-5296.

576. **Willetts N, B Wilkins.** 1984. Processing of plasmid DNA during bacterial conjugation. Microbiol. Rev. 48(1): 24-41.

577. **Williams RJ**. 2001. Globalization of antimicrobial resistance: epidemiological challenges. Clin. Infect. Dis.33 Suppl 3:S116-7.

578. **Williamson LL, Borlee BR, Schloss PD, Guan C, Allen HK, Handelsman J.** 2005. Intracellular screen to identify metagenomic clones that induce or inhibit a quorum sensing biosensor. Appl. Environ. Microbiol. 71(10):6335-6344.

579. **Wilson KH and RB Blitchington.** 1996. Human colonic biota studied by ribosomal DNA sequence analysis. Appl. Env. Microbiol. 62(7):2273-2278.

580. **Wilson GA, KF Bott.** 1968. Nutritional factors influencing the development of competence in the *Bacilus subtilis* transformation system. J. Bacteriol. 95(4): 1439-1449.

581. **Wintero AK, Fedholm M, Davies W.** 1996. Evaluation and characterisation of a porcine small intestine cDNA library analysis of 839 clones. Mammalian Genome. 7(7):509-517.

582. **Witte W.** 2004. International dissemination of antibiotic resistant strains of bacterial pathogens. Infect. Genet. Evol. 4(3):187-191.

583. **Woodford N, Warner M, Aucken HM.** 2000. Vancomycin resistance among epidemic strains of methicillin resistant *Staphylococcus aureus* in England and Wales. J. Antimicrob. Chemother. 45(2):258-259.

584. **Yamaguchi A, Adachi K, Akasaka T, Ono N, Sawai T.** 1991. Metal-tetracycline/H^+ antiporter of *Escherichia coli* encoded by a transposon Tn*10*. J. Biol. Chem. 266(10):6045-6051.

585. **Yan W.** 1991. Characterisation of erm resistance in *Camplobacter jejuni* and *Campylobacter coli*. Antimicrob. Agents Chemother. 35(10):1989-1996.

586. **Yun J, S Ryu.** 2005. Screening for novel enzymes from metagenome and SIGEX as a way to improve it. Microb. Cell Factories. 4(1):8.

587. **Zheng Z, Stewart PS.** 2002. Penetration of rifampin through *Staphylococcus epidermidis* biofilms. Antimicrob. Agents Chemother. 46(3):900-3.

588. **Zhou J, Bruns MA, Tiede JM.** 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. 62(2): 316-322.

589. **Ziha-Zarifi I, Llanes C, Kohler T, Pechere JC, Plesiat.** 1999. In vivo emergence of multidrug-resistant mutants of *Pseudomonas aeruginosa* overexpressing the active efflux system MexA-MexB-OprM. Antimicrob. Agents Chemother. 43(2):287-91.

590. **Zilhao R, Papadopoulou B, Courvalin P.** 1988. Occurrence of the Campylobacter resistance gene *tet*(O) in *Enterococcus* and *Streptococcus* spp. Antimicrob. Agents Chemother. 32(12):1793-6.

591. **Zirakzadeh A, R Patel.** 2006. Vancomycin resistant enterococci ; colonisation, infection, detection, and treatment. Mayo. Clin. Proc. 81(4) :529-536.

592. **Zoetendal EG, Plugge CM, Akkermans AD, de Vos WM.** 2003. *Victivallis vadensis* gen. Nov., sp. Nov., a sugar-fermenting anaerobe from human faeces. Int. J. Syst. Evol. Microbiol. 53(Pt 1) : 211-215.

593. **Zoetendal EG, Collier CT, Koike S, Mackie RI, Gaskins HR.** 2004. Molecular ecological analysis of the gastrointestinal microbiota: a review. J. Nutr. 134(2):465-472.

APPENDICES

Appendix 1: Composition of Buffers and Solutions

Buffer, Solution, Medium	Per Litre
LB (Luria-Bertani) medium	1.5% Tryptone (Oxoid) 0.5% Yeast extract (Oxoid) 1.0% NaCl (5.0% Technical Agar 3) (Oxoid)
DNA Extraction Buffer	100 mM Tris-HCL [pH 8.0] 100 mM sodium EDTA [pH 8.0] 100 mM sodium phosphate [pH 8.0] 1.5 M NaCl 1% CTAB
50 x TAE	242g Tris base 57.1 ml Glacial acetic Acid 18.6 g EDTA dH_2O to 1L
Hybridisation Solutions: Depurination solution Denaturation solution Neutralization solution SSC x 20 Primary wash buffer	 250 mM HCl 1.5 M NaCl 0.5 M NaOH 1.5 M NaCl 0.5 M TrisHCl pH adjusted to 7.5 0.3 M Na_3 citrate 3.0 M NaCl pH 7.0 0.4% SDS 0.5 x SSC Urea

Reduced Transport Fluid (RTF) Stock Solution 1	Dibasic potassium phosphate 0.6 g Sterile dH_2O to 100 ml
$MgSO_4$ Stock Solution	$MgSO_4$ 2.5 g Sterile dH_2O to 100 ml
Stock Solution 2	Potassium Chloride 1.2 g Ammonium Sulphate 1.2 g Monobasic potassium phosphate 0.6 g $MgSO_4$ stock solution 1.0 ml Sterile dH_2O 99 ml
Sodium Carbonate Solution	Sodium carbonate 0.8 g Sterile dH_2O 10 ml
Preparation for 100 ml:	Stock 1 7.5 ml Stock 2 7.5 ml Sodium carbonate 0.5 ml Sterile dH_2O 80 ml Autoclave, once cool add filter sterilised solution of DTT (0.02 g in 5 ml dH_2O). Solution is stable for one week.

All chemicals are from Sigma Aldrich unless otherwise stated.

Appendix 2: Profile of Subjects from whom Oral and Faecal Samples were Taken.

English Volunteers

Number	Age	Gender
S1	45+	M
S2	45+	M
S3	41-45	M
S4	36-40	F
S5	45+	M
S6	31-35	M
S7	31-35	M
S8	21-25	F
S9	26-30	F
S10	21-25	M
S11	21-25	M
S12	21-25	F
S13	36-40	F
S14	31-35	F
S15	26-30	F
S16	26-30	M
S17	31-35	M
S18	21-25	F
S19	21-25	F
S20	26-30	M

French Volunteers

Number	Age	Gender
S1	26-30	M
S2	26-30	M
S3	31-35	M
S4	21-25	F
S5	45+	M
S6	36-40	M
S7	26-30	M
S8	45+	F
S9	36-40	F
S10	21-25	M
S11	21-25	M
S12	31-35	F
S13	41-45	F
S14	21-25	F
S15	26-30	F
S16	21-25	M
S17	26-30	M
S18	26-30	F

S19	31-35	F
S20	26-30	M

Finnish Volunteers

Number	Age	Gender
S1	45+	M
S2	36-35	F
S3	35-40	F
S4	41-45	F
S5	41-45	F
S6	45+	F
S7	26-30	F
S8	26-30	M
S9	31-35	F
S10	36-40	F
S11	41-45	F
S12	41-45	F
S13	41-45	F
S14	31-35	F
S15	45+	F
S16	21-25	M
S17	31-35	F
S18	31-35	M
S19	45+	M
S20	45+	F

Italian Volunteers

Number	Age	Gender
S1	36-40	F
S2	36-40	F
S3	26-30	F
S4	31-35	M
S5	36-40	M
S6	45+	M
S7	31-35	M
S8	26-30	F
S9	36-40	M
S10	26-30	M
S11	26-30	M
S12	45+	M
S13	26-30	M
S14	26-30	F
S15	26-30	F
S16	21-25	M

S17	26-30	F
S18	26-30	F
S19	26-30	F
S20	26-30	F

Norwegian Volunteers

Number	Age	Gender
S1	21-25	F
S2	31-35	F
S3	36-40	F
S4	45+	F
S5	26-30	F
S6	31-35	F
S7	41-45	F
S8	45+	F
S9	31-35	F
S10	36-40	F
S11	26-30	M
S12	26-30	M
S13	41-45	M
S14	26-30	M
S15	26-30	M
S16	31-35	M
S17	36-40	M
S18	36-40	M
S19	45+	M
S20	41-45	M

Scottish Volunteers

Number	Age	Gender
S1	21-25	F
S2	21-25	F
S3	26-30	F
S4	26-30	F
S5	26-30	F
S6	31-35	F
S7	36-40	F
S8	36-40	F
S9	45+	F
S10	45+	F
S11	41-45	F
S12	41-45	F
S13	26-30	M
S14	26-30	M

S15	36-40	M
S16	36-40	M
S17	31-35	M
S18	41-40	M
S19	45+	M
S20	45+	M

Appendix 3: Instructions to Volunteers

Explanation of why we are taking these samples and what we will do with them

Thank you for agreeing to supply a stool and saliva sample. These will be used as part of a European Commission funded study, the major aim of which is to determine what type of antibiotic resistance genes are contained within bacteria in the human gastrointestinal tract and if these genes are likely to be able to spread to other bacteria. As you probably know there has been a recent increase in the levels of antibiotic resistant bacteria in our environment and this has consequently hampered our ability to treat diseases caused by bacteria. In order to combat this problem we need to have a deeper understanding of the mechanisms involved in transfer of these resistance genes and to know in what type of bacteria the genes reside. We also need to know if antibiotic resistant bacteria are more common in certain parts of Europe.

What will happen to the Sample?

After you have provided the stool and saliva sample a courier will transfer these to the Eastman Dental Institute in London. Here the samples will be split into two, with one sample being used to determine what type of antibiotic resistant bacteria are capable of growing under laboratory conditions. As it is well known that not all bacteria can be grown under conditions obtainable in the lab, the other half of the sample will be used to isolate DNA from all the bacteria in the sample. By using the techniques of genetic manipulation it should be possible to determine what antibiotic resistant genes are present in the total sample (both from laboratory-cultivable and non-cultivable).

Once the antibiotic resistance genes have been isolated the mechanisms used for transmission of these genes between different bacteria and the transmission of the genes between different environments will be determined. The results of this study will be published in scientific literature and at scientific meetings.

Volunteers

1). A total of 20 volunteers must be used – preferably (but not essentially) the same volunteers for both the saliva samples and the stool samples.
2). These should be healthy adults (between 18 and 60 years of age).
3). They may be male or female – ideally there should be equal numbers of each – but this is not essential. They must NOT all be exclusively male or female.
4). They must NOT have taken antibiotics during the three months prior to sampling.
5). The samples MUST be collected and couriered to the Eastman on the same day.

Faecal Specimen Collection

1). Each volunteer must pass their stool into a disposable paper container (picture). A portio of this then needs to be transferred to the faecal sample container (a plastic container with a brown lid) while wearing the disposable gloves provided.
2). The sample container is opened and small portions of the stool are transferred to the container using the small spatula attached to the lid until the container is a quarter full.
3). The quarter-full container is then given to the person responsible for collecting the specimens.
4). The person responsible for collecting the specimens must add an equal volume of Cary Blair medium – the container should now be approximately half full. This should be done in a microbioloical safety cabinet while wearing disposable gloves.
5). Replace the lid and secure tightly.
6). Ensure the container is wiped clean.
7). Label the samples with the date and time of collection.
8). Place the sample container into the plastic transportation tube (a plastic tube containing absorbant paper). Please note that the base of the faecal sample container is slotted into the lid of the transport container to prevent the movement of the sample container during transport.
9). Place each of the twenty transport containers into the box supplied by the courier (DHL).
10). Courier the samples to the Eastman to arrive before 12am the following day. Email the courier reference to the Eastman l.Seville@eastman.ucl.ac.uk

Paraffin Wax Stimulated Saliva Collection

1). Each volunteer places a 1g piece of paraffin wax into their mouth and chews.
2). At intervals the volunteers leans forward and spits into the sterile saliva collection container (avoid dribbling as this will result in sample contamination with bacteria present on the lips and chin).
3). After 5 mls have been collected, the lid should be replaced and secured tightly.
4). The container is then wiped clean and given to the person responsible for collection of the specimens.
5). The container is opened (in a microbiological safety cabinet) and an equal volume of reduced transport fluid should be added (this transport fluid has a limited life-time

and will be sent to each centre on the Monday of the week in which the samples are to be taken).
6). Replace the lid and secure tightly.
7). Ensure that the container is wiped clean.
8). Label the sample with the date and time of collection.
9). Place each sample container into the plastic transportation tube (a plastic tube containing absorbant paper). Please note that the base of the saliva sample container is slotted into the lid of the transport container to prevent the movement of the sample container during transport.
10). Place each of the twenty transport containers into the box supplied by the courier (DHL).
11). Courier the samples to the Eastman to arrive before 12am the following day. Email the courier reference to the Eastman l.Seville@eastman.ucl.ac.uk

Appendix 4: Primers used in this study

Primer Name	Primer Sequence	Reference
16S rRNA gene		
27F	5'-AGA GTT TGA TCM TGG CTC AG-3'	Lane, 1996
357F	5'-CTC CTA CGG GAG GCA GCA G-3'	
1492R	5'-TAC GGY TAC CTT GTT ACG ACT T-3'	
Tet determinants		
tet(M)F	5'-GTG GAC AAA GGT ACA ACG AG-3'	Ng, 2001
tet(M)R	5'-CGG TAA AGT TCG TCA CAC AC-3'	Ng, 2001
tet(O)F	5'-AAC TTA GGC ATT CTG GCT CAC-3'	
tet(O)R	5'-TCC CAC TGT TCC ATA TCG TCA-3'	Ng, 2001
tet(S)F	5'-CAT AGA CAA GCC GTT GAC C-3'	Melville *et al.* 2001
tet(S)R	5'-ATG TTT TTG GAA CGC CAG AG-3'	
tet(32)F	5'-GAA CCA AGA TGC TGC TCT T-3'	Roberts et al. 2006
tet(32)R	5'-CAT AGC CAC GCC CAC ATG AT-3'	
tet(S)F complete gene	5'-ATA AAG AAT CCC TTA TCA AC-3'	Aminov, 2001
tet(S)R complete gene	5'-TTA TAA AGG ATA TCA AGA AC-3'	
Universal RPP-F	5'-GGM CAY RTG GAT TTY WTI GC-3'	
Universal RPP-R	5'-TCI GMI GGI GTR CTI RCI GGR C-3'	
Tn*916*		
*Intxis*1	5'-CGC CAA AGG ATC CTG TAT ATG-3'	Roberts *et al.* 2001
*Intxis*2	5'-GCT GTA GGT TTT ATC AGC TTT TGC-3'	
RT1	5'-CTC TAT CCT ACA GCG ACA GC-3'	Roberts (thesis)
RT2	5'-ATA TAC GAG TTT GTG CTT GT-3'	
RT3	5'-CCT GCT CGG TGT ATT CAA GA-3'	Roberts (thesis)
RT4	5'-TCT TTG CGT CTG GCT CTG TA-3'	
M13F	5'-CGC CAG GGT TTT CCC AGT CAC GAC-3'	Invitrogen
M13R	5'-AGC GGA TAA CAA TTT CAC ACA GGA-3'	
pCC1BAC-F	5'-GGATGTGCTGCAAGGCGATTAAGTTGG-3'	Epicentre
pCC1BAC-R	5'-CTCGTATGTTGTGTGGAATTGTGAGC-3'	

Appendix 5: Antibiotic Working/Stock Concentrations.

Antibiotic (all Sigma Aldrich)	Solvent (100% AnalaR BDH diluted with dH_2O)	Stock Concentration	Working concentration
Tetracycline	50% ethanol	10 mg/ml	2 µg/ml for screening for resistant isolates. 10 µg/ml for screening BAC clones
Erythromycin	70% ethanol	10 mg/ml	1 µg/ml for screening isolates
Vancomycin	Sterile distilled water	10 mg/ml	8 µg/ml for screening isolates
Ampicillin	1M Sodium hydroxide	100 mg/ml	50 µg/ml for selection of pUC19
Spectinomycin	70% ethanol	1000 mg/ml	50 µg/ml for selection of pDL278 in *E. coli* 800 µg/ml for selection of pDL278 in *S. mutans*
Chloramphenicol	100% ethanol	12.5 mg/ml	12.5 µg/ml for selection of pCC1BAC

Appendices 6, 7 and 8 are on the CDRom provided in the front cover of this thesis

Appendix 9: SFtetC10 (tet(M) BAC Clone) End-Sequencing Results

>SFtC10_pCC1F_D10 sequence exported from chromatogram file
CGACGTTGTAAAACGACGGCCAGTGNATTGTAATACGACTCACTATAGGGCGAATTCGAG
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GACCGTCTTTGGCAAGCAGCCGTTCCATTTCGTTGAACGCCGCTCAAATTTTCTGTTTGCTC
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>SFtC10_pCC1R_E10 SEQUENCE EXPORTED FROM CHROMATOGRAM FILE
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Appendix 10: NFtetC1 (tet(O) BAC Clone) Insert Sequence

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Appendix 11: IStetC2 (tet(A) / unknown BAC Clone) Subclone Sequence data

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AGTCCGGTTCTTCAGGCATTCACCACTTGCACGTCTGAACAGCATTTCAAGAATGGCAAA
CTTCTTGTTTTTATCAAAAGAAGATATGGGATCATCAAGGATAATCAGTCCCGGGTTTTT
TGACAGGCATTCATACATGAATAACACAATCGCAAAGGCGTTCCGTTCTCCATAGCTGAG
ATGCTGGCTACCGCCGCTGACGTACCCGTCAAAATCTATGTGTCGTAGCCTGAGTTTTCT
CTGCTCTCCCTCGCCGGCTATATCCACCCTGTATTTATAGCCTGCATAAGTGAGGAAATT
ATTGATGTTTGTTTTGTGCTGTGCGATCAGCCGGATCATGCTGTCCCGGTGCCGGTTAAT
TTTTCCCTGCAGCGGTCCGGCAAGGTTTATCAGGTCCATAAGGGCCGCGTTCAGTCTGTC
GGTGATCCCCTGCATGAGTTCGGATTGCAGATCAGGGAAGAACTGCAGGTCAATCAACCT
GGCAGTGAGCACCTCCCGAACGTTCTGCTGTTCCTGCAGGCTGAAAACATTCAGGCCTCT
CAGCGCAGTGAGTTTCTCTGTCAGCGTATCGGTCTGGCGTTTCAACGCCACTAGGTACTC

GATATGTTCGGCTTCCGGACCGTTCTGAAGCATAGTAATGGCCAGAAGGCGTTCTCTGGC
GCTCTCCGTCAGGTAATTACCGAGGTTTTCCACCAGTCTGATGATAGCGGTCAGGTTTTT
AATGGTGGATTTATCGTATTCCTCTCTGACTTGGCGAATTTGTGCTTCCTTTCCCGTGAT
ATCACCGGTACAGAACGGACAGCAGCCATCCGAAAGGGGCGAAAACTCGAGTCCCTTTGT
CTGCCAGTCAATCCATTCCACGCGGCGTTCACTGCGGATGTAGGGTTGATAGTTCTCAAG
ACCAGCCGGAATGTGGTGTATTTTATTTCCGCCTGACAGACCTCTCATGCCGGTTGAAGA
ACGTGAAATCCCGGAACTGGTTGATCTGAAAGCATTACTGAGTTCCTGTAGATGGTCTAT
CAGAGAGTTCAGCTCAGTGTGATCCGTGAAAACAGCCCGGATCTTTTGCGTCATTTCTTC
TATTTCACGCTCTCTTTCCGCATGGGCTTGATTGCGGATAAGAATATTAAAGCTATCACT
GATGAGCTCGTCCGGTTGGAACGTAAACTGACTGACGTACTCCTCGTTGAAGCACATCAC
GTCCCCAATCCCGTCAGCACCGATGACAATGGGCCCGGTGCTATCGGGGTTTTCTCCGCG
CAGCCGGAAGGGCATCAGTGCCTGTAGTCCCTGAATGTCGTCCCGCGCCGCACAGCTTAT
GGCCCGTGACAGCGTACTTTTTCCAGTACCATTTGGCGCAAACTTAATGTTCAGTTTATC
GGCAGTGAGGGTGATATGTGCTCGACGATGTTATTGCAGGGGCGATTTCAATGTCCATTT
ATCTGTCCCTTGGCCAGTTATTCCGGGGGGATTCATATCCTTCCGCAAAATGGGCCCGCA

Appendix 10: CLUSTAL W multiple sequence alignment of TnpV/Tet(O) BAC clone with TnpV sequences from BAC end-sequencing results (chapter four) and TnpV sequences from the databases.

```
BACTnpV1          ---------------------------------PLGHYGRLRKAYLEMHRPILFNELVLS 27
BACTnpV3          ---------------------------------PLGHYGRLRKAYLEMHRPILFNELVLS 27
BACTnpV6          ---------------------------------PLGHYGRLRKAYLEMHRPILFNELVLS 27
CcoliTnpV         ---------MNITYTQNGDYLIPNIII--RKTKPLGHYGRLRKAYLEMHRPILFNELVLS 49
BACTnpV4          -------------------------------------YGRVRKAYLEMRRPILFDELVLS 23
BACTnpV5          ---------------------------------PLGHYGRVRKAYLEMRRPILFDELVLS 27
BACTnpV2          ---------------------------------PLGHYGRLRKAYLEMRRPILFDELVLS 27
Tn4453TnpV        MQRFITDERTGIRYELIGDYYYPCLTA--EEKPLLSRYGRMRERYLKEHKRVLYYTLMTS 58
Tn4451TnpV        MQRFITDERTGIRYELIGDYYYPCLTA--EEKPLLSRYGRMRERYLKEHKRVLYYTLMTS 58
BACTnpVClone      ----------------------------------------HLDYLREYRKITYTNLLTS 19
SpyogenesTnpV     -MAKSLFEELGGKYERQGDYLIPCLTVPAEEEQAIGIWGQRHLDYLKQYRKVTYTNLLTS 59
                                                          :  **.  : : :  *: *

BACTnpV1          DKLFEHCAEIDEAARNRMELIVRSLAEQNGVTEQLKAKNQM-------ACGAADEQ---- 76
BACTnpV3          DKLFEHCAEIDEAARNRMELIVRSLAEQNGVTEQLKAKN---------------------- 66
BACTnpV6          DKLFEHCAEIDEAARNRMELIVRSLAEQNGVTEQLKAKNQM-------EWVRQTSS---- 76
CcoliTnpV         DKLFEHCAEIDEAARNRMELIVPELVKRNGVTEQLKAENQMEWVRQMNACKAQAEEVVKA 109
BACTnpV4          DKLFERCGEIEEAARNRMELIARALADQNGGT-QLGAKNQM-------GWVR-------- 67
BACTnpV5          DKLFERCGEIEEAGRNRMELIVRALADQNGVT-QLKAKNQM-------GWVR-------- 71
BACTnpV2          DKLFERCGEIDEAGRNRMELIVRALADQNGGT-QLKAKNQR-------GWVR-------- 71
Tn4453TnpV        GKLYEHLAEIDTSACDMAEYLIKEMARKQGVTEQLKAVDMMRWIGLMNNIRACVDEIVLN 118
Tn4451TnpV        GKLYEHLAEIDTSACDMAEYLIKEMARKQGVTEQLKAVDMMRWIGLMNNIRACVDEIVLN 118
BACTnpVClone      GRLNAYLADIDRQAQEHFERLIEGMKQAQGITECLKEENALEWTGRTNNIRACAREIVEK 79
SpyogenesTnpV     GRLNAYLADINRQVQERFERLIEGMKQAQGITEQLKAENALEWTGYLNNIRACAREIVEK 119
                  .:*    .:*:    :  * :   :   :* *  *   :

BACTnpV1          -----
BACTnpV3          -----
BACTnpV6          -----
CcoliTnpV         ELIYD 114
BACTnpV4          -----
BACTnpV5          -----
BACTnpV2          -----
Tn4453TnpV        DIVYS 123
Tn4451TnpV        DIVYS 123
BACTnpVClone      EIIFA 84
SpyogenesTnpV     EIIFA 124
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Characterization of the Ends and Target Site of a Novel Tetracycline Resistance-Encoding Conjugative Transposon from *Enterococcus faecium* 664.1H1